

THE REGULATION OF STARCH  
BIOSYNTHESIS IN DEVELOPING  
WHEAT ENDOSPERMS

HARRY LIONEL RIFFKIN

DOCTOR OF PHILOSOPHY  
UNIVERSITY OF EDINBURGH

1987



To my wife Mary and daughter Rebecca

## DECLARATION

This thesis was composed by myself and describes my own original work and has not been submitted for a degree at any other University.

## CONTENTS

Abstract

Abbreviations

Acknowledgements

1.0 General Introduction	1
1.1 Production and uses of wheat	1
1.2 General morphology of wheat caryopsis development	3
1.2.1 Floral components of the wheat plant	3
1.2.2 Genetic make-up of the wheat caryopsis	5
1.2.3 Caryopsis development	6
1.3 Physiological factors related to grain filling	9
1.4 Carbohydrate precursors for endosperm starch biosynthesis	12
1.4.1 Carbohydrate metabolism in relation to endosperm starch biosynthesis	15
1.5 Starch synthesis	17
2.0 Defined growth characteristics of wheat cv. Sicco	21
2.1 Introduction	21
2.2 Methods	22
2.2.1 Plant material	22
2.2.2 Sampling methods	23
2.2.3 Fresh and dry weight determinations	23
2.2.4 Morphological changes during development	23
2.2.5 Presentation of data	25
2.3 Results	25
2.3.1 Determination of anthesis	25
2.3.2 Changes in fresh weight, dry weight and moisture content	29
2.3.3 Changes in physical dimensions and tissue morphology	34



2.4 Discussion	36
2.4.1 Determination of anthesis	36
2.4.2 Growth characteristics - grain filling	37
2.4.3 Changes in morphology during caryopsis, endosperm and embryo development	41
2.5 Conclusions	42
 3.0 Determination of some of the metabolites implicated in sucrose-starch conversion in developing wheat endosperms	44
3.1 Introduction	44
3.2 Methods	45
3.2.1 Plant material	45
3.2.3 Metabolite analysis	46
3.2.3.1 Preparation of endosperm extracts	46
3.2.3.2 Determinations of sugars and sugar phosphates	47
3.2.3.3 Ethanolic extracts	50
3.2.3.4 Determinations of nucleoside diphosphate sugars and nucleoside di- and triphosphates	51
3.2.3.5 Measurement of soluble protein	52
3.2.3.6 Analysis of data	56
3.3 Results	56
3.3.1 Metabolite determinations	56
3.4 Discussion	60
3.4.1 Sampling of plant material and presentation of data	60
3.4.2 Metabolite determinations	61
3.5 Conclusions	65
 4.0 Sucrose synthase and invertase in developing wheat endosperms	67
4.1 Introduction	67
4.2 Methods	68
4.2.1 Plant material	68
4.2.2 Reagents	68

4.2.3	Time course study of sucrose synthase and invertase activities	69
4.2.4	Preparation of enzyme extracts for enzyme studies	69
4.2.5	Determination of sucrose synthase activity	69
4.2.6	Determination of invertase activity	70
4.2.7	Linearity of response	70
4.2.8	Determination of sucrose synthase and invertase activity in pericarp/testa tissue	71
4.2.9	Analysis of soluble protein	71
4.2.10	pH-dependent sucrose synthase activity	71
4.2.11	pH-dependent pericarp/testa invertase activity	72
4.2.12	Enzyme kinetics	72
4.2.13	Inhibition studies	73
4.2.14	Presentation of data	76
4.3	Results	76
4.3.1	Sucrose synthase and invertase activities during endosperm development	76
4.3.2	pH-dependent activity of endosperm sucrose synthase and pericarp/testa invertase	78
4.3.3	Determinations of $K_m$ for UDP- and sucrose - dependent endosperm sucrose synthase initial reaction velocities	82
4.3.4	Endogenous inhibitor studies	85
4.4	Discussion	86
4.4.1	Developmental studies	86
4.4.2	UDP- and sucrose-dependent sucrose synthase initial reaction velocities	90
4.4.2.1	Presentation of data	90
4.4.2.2	UDP-dependent initial reaction velocities	91
4.4.2.3	Sucrose-dependent initial reaction velocities	93
4.4.3	Endogenous inhibitor studies	93
4.5	Conclusions	94

5.0 ADP- and UDP- glucose pyrophosphorylases in developing wheat endosperms	96
5.1 Introduction	96
5.2 Methods	97
5.2.1 Plant material	97
5.2.2 Reagents	97
5.2.3 Tissue extracts for enzyme studies	98
5.2.4 Enzyme assays	99
5.2.4.1 ADP-glucose pyrophosphorylase (EC 2.7.7.27), GlP-dependent activity	99
5.2.4.2 ADP-glucose pyrophosphorylase, PPi-dependent activity	102
5.2.4.3 UDP-glucose pyrophosphorylase (EC 2.7.7.9), GlP-dependent activity	102
5.2.4.4 Control enzyme assays	103
5.2.5 Product identification	108
5.2.6 Ammonium sulphate fractionation of endosperm extracts	110
5.2.7 Ammonium sulphate fractionation of leaf extracts	112
5.2.8 Measurement of enzyme activity in ammonium sulphate fractions	112
5.2.9 Gel filtration chromatography	112
5.2.10 Ion-exchange chromatography	113
5.2.11 Enzyme studies on ADP- and UDP- glucose pyrophosphorylases from endosperm and leaf tissue	114
5.2.11.1 pH profiles	115
5.2.11.2 Reaction linearity	115
5.2.11.3 Endosperm ADP-glucose pyrophosphorylase activity as a function of time after dialysis	116
5.2.11.4 Thermal characteristics of the endosperm enzyme	116
5.2.11.5 Enzyme kinetics	116
5.2.11.6 Metabolite activation/inhibition	117

5.2.11.7	The effect of ADP-glucose, PPi and Pi on the activity of endosperm ADP-glucose pyrophosphorylase	118
5.2.11.8	The effect of $\text{MgCl}_2$ , BSA and DTE on endosperm ADP-glucose pyrophosphorylase activity	118
5.2.11.9	Effect of various salts on endosperm ADP-glucose pyrophosphorylase activity	119
5.3	Results	119
5.3.1	The radiochemical assay ( $[U - ^{14}\text{C}]$ ) for ATP- and UTP-dependent pyrophosphorylases	120
5.3.2	ADP- and UDP-glucose pyrophosphorylase activities during endosperm development	123
5.3.3	Ammonium sulphate fractionation	127
5.3.4	Linearity of the radiochemical assay for ADP- and UDP-glucose pyrophosphorylases	129
5.3.5	pH-dependent activity	132
5.3.6	Thermal characteristics of endosperm ADP- and UDP-glucose pyrophosphorylases	132
5.3.7	Gel filtration and ion exchange chromatography	134
5.3.8	Time dependent activity of ADP-glucose pyrophosphorylases	137
5.3.9	Metabolite activation or inhibition of endosperm and leaf ADP-glucose pyrophosphorylases	137
5.3.10	Determinations of $K_m$ for endosperm and leaf ADP-glucose pyrophosphorylase initial reaction velocities	139
5.3.12	The effect of ADP-glucose, PPi and Pi on the activity of endosperm ADP-glucose pyrophosphorylase	152
5.3.13	The effect of $\text{MgCl}_2$ , DTE and BSA on endosperm ADP-glucose pyrophosphorylase activity	152
5.3.14	Activity of endosperm ADP-glucose pyrophosphorylase in the presence of various salts	154

5.4 Discussion	157
5.4.1 The radiochemical assay for the determination of ADP- and UDP-glucose pyrophosphorylase activities	157
5.4.2 Developmental studies	158
5.4.3 Studies on endosperm and leaf pyrophosphorylases	162
5.5 Conclusions	177
6.0 General conclusions and suggestions for future work	179
Bibliography	184

## ABSTRACT

The purpose of this work is to identify some of the mechanisms regulating the conversion of assimilates to starch during endosperm development in wheat.

Since environmental conditions affect the rate of grain development, a standard system for ageing developing caryopses was devised. The system was based on the morphological changes accompanying caryopsis development, under field conditions, from anthesis to harvest-ripeness. Accordingly, because the wheat caryopsis passes through similar developmental changes which occur at approximately the same relative time regardless of the time scale, the tissues of the caryopsis, e.g. the endosperm, could be standardised according to this system. This ensured that tissues at the same stage of development could be compared, even when grown under different environmental conditions, e.g. under glass.

Sucrose is the principal transported sugar in wheat and was found to be the most abundant sugar in developing wheat endosperms. Glucose and fructose were present in lower amounts and in different relative quantities, with glucose declining throughout endosperm development. The higher quantities of fructose were consistent with low levels of endosperm invertase activity and much higher activities of endosperm sucrose synthase.

Endosperm sucrose synthase activity reached an apparent maximum catalytic rate during the period of rapid dry weight accumulation. The levels of UDP in developing endosperms also reached a maximum at this time. The curve of UDP-dependent sucrose synthase initial reaction velocities was sigmoidal, thus endosperm UDP levels may regulate the catabolism of sucrose.

Levels of ADP in developing wheat endosperms were higher than UDP but UDP-glucose was present in amounts approximating to twice those of ADP-glucose. This implies that ADP-dependent sucrose synthase activity was not predominant in the catabolism of sucrose in developing wheat endosperms.

Hexose sugars require to be in the form of hexose phosphates prior to further metabolism by either glycolytic enzymes or ADP- and/or UDP-glucose pyrophosphorylases. G6P was present in consistently higher amounts than either G1P or F6P suggesting rapid phosphorylation of glucose by hexokinase. Levels of G1P rose to a maximum during endosperm dry weight accumulation and were quite adequate to account for the measured rates of maximum velocity for endosperm ADP-glucose pyrophosphorylase. G1P may have been formed by the catabolism of UDP-glucose by the PPi-dependent UDP-glucose pyrophosphorylase reaction. This enzyme activity was found to be 5-7 times higher in developing wheat endosperms than either sucrose synthase or ADP-glucose pyrophosphorylase.

Differences were observed in the properties of ADP-glucose pyrophosphorylases from endosperm and leaf tissues suggesting that the control mechanisms differed between tissues. Both enzymes were partially purified by ammonium sulphate fractionation and the precipitates stored in 85 per cent ammonium sulphate. The endosperm enzyme was stable in the unfractionated extract and in the stored precipitate but, on subsequent dialysis of the stored precipitate, rapidly lost activity, with a half-life of about 4h. The dialysed activity was dependent on  $MgCl_2$  and was partially stabilised by Pi but was not activated by 3-PGA. The leaf enzyme was stable to fractionation by ammonium sulphate and to storage and dialysis but both the crude tissue and partially purified activities were dependent on the presence of 3-PGA.

PPi was found to be an efficient inhibitor of endosperm ADP-glucose pyrophosphorylase while Pi was not, implying that PPi must be removed from the site of ADP-glucose synthesis or hydrolysed. If the generation of G1P for ADP-glucose synthesis is a result of PPi-dependent catabolism of UDP-glucose then PPi/Pi metabolism may regulate starch biosynthesis in developing wheat endosperms.

## ABBREVIATIONS

### Chemicals

ADP-glucose	adenosine diphospho - $\alpha$ -D-glucose
BSA	bovine serum albumin
DHAP	dihydroxyacetone phosphate
DTE	dithioerythritol
F	fructose
F6P	fructose-6-phosphate
F-1, 6-bisP	fructose-1, 6-bisphosphate
F-2, 6-bisP	fructose-2, 6-bisphosphate
G	glucose
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G-1, 6-bisP	glucose-1, 6-bisphosphate
GAP	glyceraldehyde-3-phosphate
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid
$\alpha$ KG	$\alpha$ -ketoglutaric acid
MES	2-[N-morpholino] ethane sulphonic acid
MOPS	3-[N-morpholino] propane sulphonic acid
PBD	2-phenyl-5-(4-biphenyl)-1,3,4-oxadiazole
PCA	perchloric acid
PEP	phosphoenol pyruvate
2-PGA	2-phosphoglyceric acid
3-PGA	3-phosphoglyceric acid
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
POPOP	1,4-di-(2-(5-phenyl oxazolyl))-benzene
tricine	(N-tris[hydroxymethyl] methyl - glycine; N-[2-hydroxy -1, 1-bis (hydroxy methyl) ethyl]-glycine)
UDP-glucose	uridine diphospho- $\alpha$ -D-glucose



### Statistics

r	sample correlation coefficient
$r^2$	sample coefficient of determination
SD	standard deviation
SEM	standard error of the mean

### Enzyme kinetics

$K_m$	Michaelis constant
s	substrate concentration
V	maximum initial reaction velocity
$v_o$	initial reaction velocity

All other abbreviations (chemical) in the text are the generally accepted abbreviations, e.g. ATP, EDTA, DEAE-cellulose, and have been used without definition as per the policy of the Biochemical Journal (The Biochemical Society, 1987).

## ACKNOWLEDGEMENTS

I would like to thank my supervisors Drs Carol M Duffus and Ian C Bridges for their constant advice and encouragement both throughout this investigation and in the preparation of this thesis. I am especially grateful to all of the staff of the Agricultural Biochemistry Department at the Edinburgh School of Agriculture for their help in carrying out this work. In particular my thanks go to Drs Tom Acamovic, Pat Cochrane and Pat Watson whose advice and training contributed , not in a small way, to the prosecution of this study.

I would also like to acknowledge the technical assistance and instruction given by Mr Geoff Foxon and Dr Peter Keeling and the co-operation of all of the staff at the ICI Corporate Bioscience Group, Runcorn, Cheshire.

I am indebted to the staff of the Crop Production Glasshouse Unit and to Dr Robert Lang and his staff at the Crops Department, Bush Estate, Midlothian for the constant supply of plant material.

Dr Steven Fry of the Botany Department; Edinburgh University, Dr Wendy Berndt, Mr Willie Rowbottom and Mr Albert Scott all of the Central Analytical Laboratories, Edinburgh School of Agriculture; Mrs Sandra Bruce at the Department of Molecular Biology, Edinburgh University; Drs Milton Park and Colin Slaughter of Heriot-Watt University and Ms Pamela Geddes and Mrs G Johnstone of Pentlands Scotch Whisky Research Ltd, Edinburgh all gave assistance at various times during this work and their help is gratefully acknowledged.

Finally I would like to thank Miss Helen Glover for her skilful typing of this thesis and my wife Mary for her assistance in proof-reading the manuscript.

This work was supported by a grant from the Science and Engineering Research Council and by Imperial Chemical Industries Plc., Runcorn, Cheshire.

## CHAPTER ONE

1.0 General Introduction1.1 Production and uses of wheat

The wheat plant is a member of the botanical family of grasses known as Gramineae. Other agriculturally important members of this family are listed in table 1.1 together with their respective world production figures including area harvested.

Table 1.1 Major cereal crops (FAO production yearbook, 1984)

<u>Cereal</u> <u>crop</u>	<u>Area harvested</u> <u>(hectares x 10<sup>-8</sup>)</u>	<u>World production</u> <u>(metric tonnes x 10<sup>-8</sup>)</u>
Wheat	2.3	5.2
Rice	1.5	4.7
Maize	1.3	4.5
Barley	0.8	1.7
Sorghum	0.5	0.7
Oats	0.3	0.4
Millet	0.4	0.3
Rye	0.2	0.3

All the grasses listed in table 1.1 are grown primarily for their seeds and are more generally referred to as cereals.

Wheat is grown essentially as a starch crop, although it is its unique blend of starch and protein that makes it important in the manufacture of baked products (Lineback, 1984). In addition to baked products, wheat starch is used as an adhesive and for sizing in the paper industry, as sizing in the textile industry and as a thickening agent in the food industry. Wheat starch is also utilised in animal feed and in production of potable alcohol. This latter usage occurs mainly in Europe, where in 1981 grain in 'free

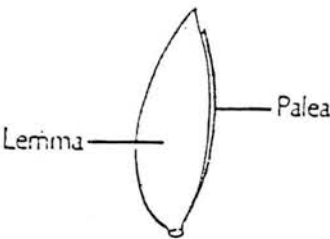


(a) ear of wheat

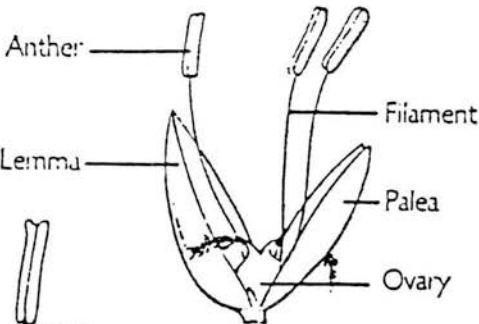


(b) individual spikelet

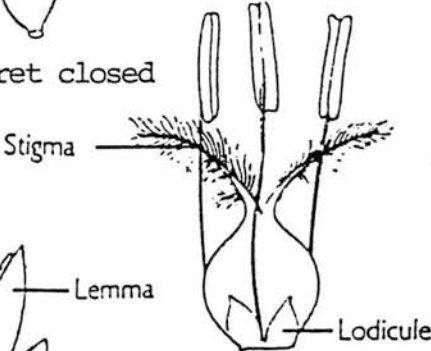
( after Watson, 1985 )



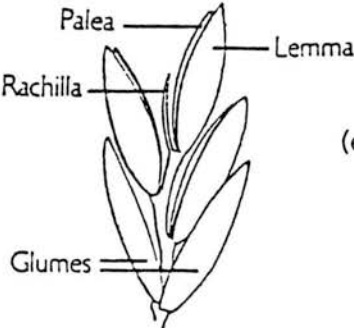
(c) single floret closed



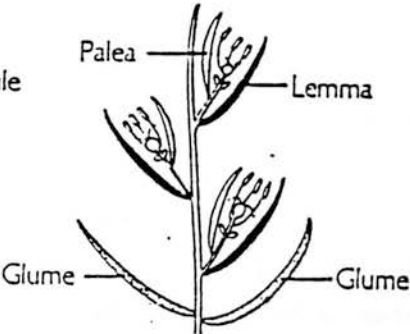
(d) single floret open



(e) floral parts



(f) spikelet



(g) diagram of spikelet

( after Langer, 1979 )

circulation' within the EEC became eligible for export refunds. The consumption of wheat starch for alcohol production has been largely at the expense of maize starch and there is now considerable interest in improving the yields of starch in European wheats.

In order to improve the yields of wheat starch it is essential that the mechanisms controlling the deposition of reserve carbohydrate in wheat be clearly understood. Presently we do not have such an understanding and as long as this situation exists it is not possible to make proper use of modern genetic techniques which might be useful in improving crop productivity (Nasyrov, 1978). The major aim of this current project is therefore to investigate some of the biochemical features that may regulate starch biosynthesis in wheat.

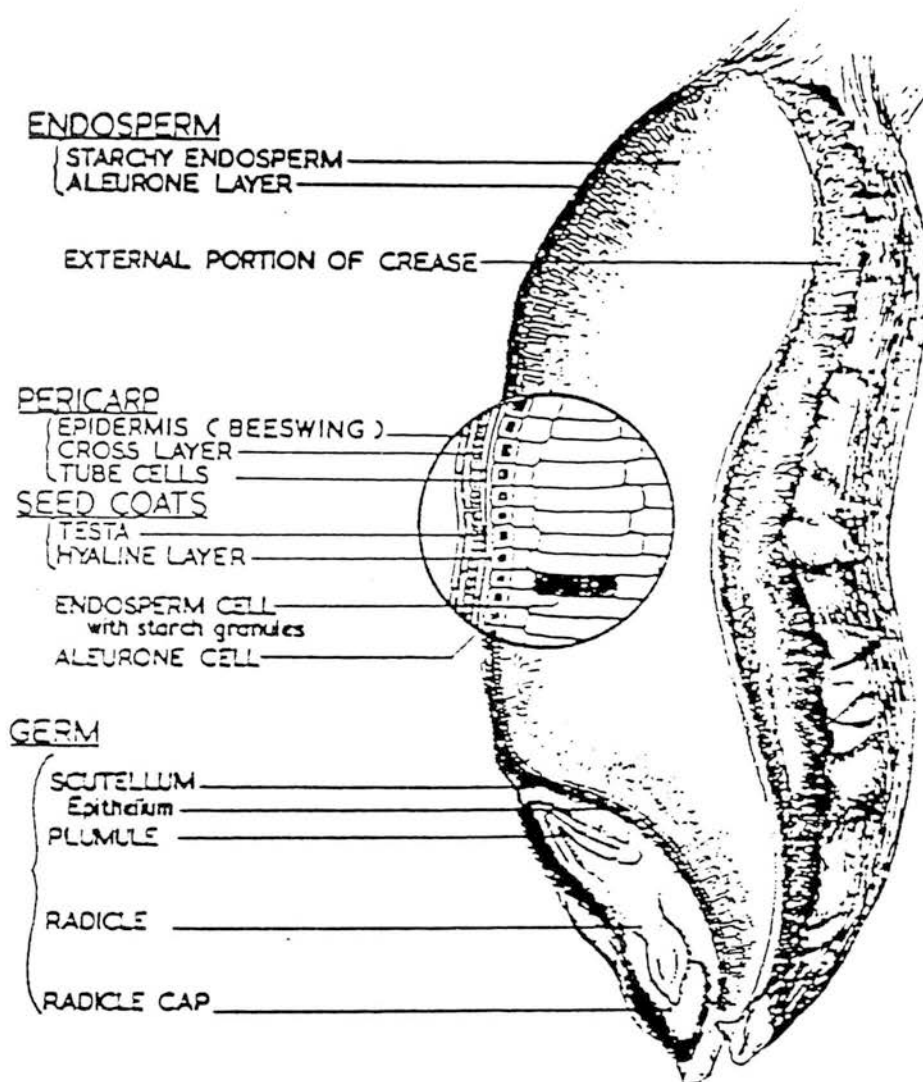
## 1.2 General morphology of wheat caryopsis development

In order to study the regulation of starch biosynthesis in wheat, or indeed any cereal crop, it is necessary to define, clearly, the plant tissues which are to be investigated. Since cereal yields relate specifically to seed production, this section will give a general description of the floral parts, tissues and morphology pertaining to seed development. For a more detailed description, the reader is referred to recent reviews by Simmonds and O'Brien (1981) and Duffus and Cochrane (1982). The classical work on the growth and development of the wheat plant is that of Percival (1921) although copies of this monograph are now relatively rare.

### 1.2.1 Floral components of the wheat plant

The inflorescence or ear of wheat (fig 1.1a) is composed of a main rachis bearing two opposite rows of lateral secondary spikelets and a single fertile terminal spikelet. Each spikelet (fig 1.1b) consists of a rachilla (fig 1.1f) which bears varying numbers of florets (3-9 depending on cultivar). The rachis of the main axis bears approximately 20-30 spikelets. At the base of each spikelet

Figure 1.2 Diagram of a longitudinal section of a wheat caryopsis through crease and embryo ( after Kent, 1983a)



(fig 1.1f) occurs a pair of bracts known as the glumes with each floret composed of a pair of flowering glumes known as the palea and lemma (figs 1.1c,d,f and g), three stamens, two styles terminating in feathery stigmas and an ovary containing a single ovule (figs 1.1d and e). The lodicules (fig 1.1e) swell up during flowering, pushing apart palea and lemma, thus allowing the anthers and stigmas to emerge (anthesis). Later the lodicules collapse and the flowering glumes close again.

### 1.2.2 Genetic make-up of the wheat caryopsis

A primary feature of the seed or grain of the wheat plant (fig 1.2) is that it is not truly a seed but the ripened ovary of a flower comprising the fruit coat (pericarp) and seed. The seed is composed of the seed coat (testa), hyaline layer (nucellar epidermis), germ (embryo) and the endosperm. During the development process the testa becomes fused to the inner layers of the pericarp and the dry indehiscent fruit is termed a caryopsis.

The genetic origin of the tissues of the wheat caryopsis requires to be correctly defined since the synthesis of enzyme systems, which may be related to starch deposition, will be governed by the genetic make up of the relevant tissues.

The tissue of the pericarp is clearly maternal tissue and enzyme systems pertaining to the pericarp should have characteristics akin to the mother plant. Prior to fertilisation, the embryo sac lies embedded within nucellar tissue which is surrounded by two layers of integuments and the ovary wall (Duffus and Cochrane, 1982). All these embedding tissues are of maternal origin. Within the embryo sac there is a number of haploid nuclei which derive from the mother plant. These female nuclei are genetically identical. Following the shedding of pollen from the anthers at anthesis and the penetration of the embryo sac by the pollen tube, a haploid male nucleus, derived from the generative nucleus of a single pollen grain, fuses with the egg nucleus to form the diploid

zygote. This diploid zygote is destined to become the embryo of the harvest-ripe caryopsis. A second haploid male nucleus fuses with two female haploid nuclei to form the triploid endosperm nucleus. Thus the embryo is formed from a diploid fusion nucleus containing one set of chromosomes from each parent, while the endosperm develops from a triploid fusion nucleus possessing three sets of chromosomes, two derived from the maternal and one from the paternal parent (Percival, 1921; Mares et al., 1975; Simmonds and O'Brien, 1981). The consequences of these events are such that within the developing caryopsis there are three genetic combinations relative to three main tissue types i.e. the pericarp (also, the testa and the nucellar layer), the embryo and the starchy endosperm (including the aleurone layer). In the harvest-ripe caryopsis, the starchy endosperm comprises approximately 83 per cent of caryopsis dry weight (MacMasters et al., 1971) and about 70 per cent of the endosperm dry weight is composed of starch (Kent, 1983b.) The major part of caryopsis starch is accounted for by endosperm starch. Clearly, since the endosperm is the repository of reserve starch, the regulation of its biosynthesis may be associated with the enzyme systems of that tissue. Given the genetic make-up of the tissues of the wheat caryopsis, it therefore makes sense to investigate the endosperm enzyme systems in isolation from the other tissues of the caryopsis.

### 1.2.3 Caryopsis development

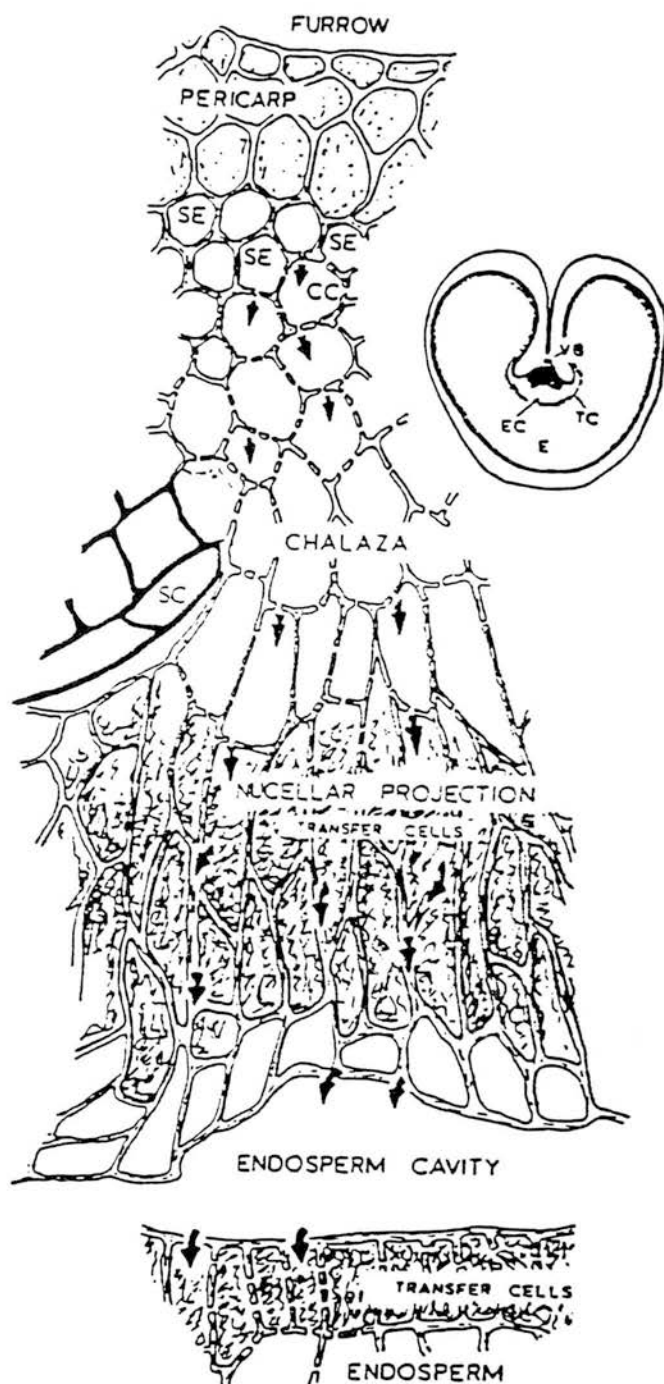
Following fertilisation, the triploid fusion nucleus of the endosperm undergoes rapid synchronous mitosis (about 10 cycles) but without concomitant cell wall formation. At the end of this period (approximately 3 days after anthesis) a single layer of nuclei lines the embryo sac, except on the ventral side where a double layer extends along the centre of the grain. This double layer will eventually form the crease region of the developing endosperm. The process of endosperm cellularisation then takes place, although this process is not clearly understood. The peripheral nuclei



continue to divide, providing nuclei for the innermost cells of the developing endosperm. Cellularisation is complete by 4-5 days after anthesis and immediately after this has occurred cell division takes place throughout the endosperm for a short period only (P. Cochrane, personal communication). Subsequently, cell division is confined solely to the peripheral layer, except for the double layer of cells in the crease region which does not participate in this meristematic activity (Evers, 1970; Simmonds and O'Brien, 1981). Peripheral cell division in wheat endosperms is thought to continue until 16-20 days after anthesis (Briarty et al., 1979).

During the period of endosperm cellularisation and cell division the maternal tissues of the caryopsis undergo a series of developmental changes. Prior to fertilisation, the pericarp is composed of thin-walled parenchyma cells about 10-16 cells across and bounded on each side by an epidermal layer. Following fertilisation the pericarp begins to differentiate into 2 main layers. The inner epidermal cells elongate longitudinally and become widely separated as the caryopsis expands. They appear in the harvest-ripe caryopsis as a series of curving cells known as tube cells. The parenchymatous layer adjacent to these inner epidermal cells elongates transversely, forming the layer known as the cross cells. These cross cells are one cell thick and are green in colour. The cells immediately outside the cross cells begin to disintegrate, eventually forming an air space which delineates the pericarp into 2 distinct layers. This air space persists until the later stages of caryopsis development. The outer layer, i.e. those parenchyma cells lying between the air space and the outer pericarp epidermis, contain starch granules some 1 - 2  $\mu$  m in diameter. The presence of these granules conveys a whitish appearance to these outer subepidermal cells. The granules persist until just after the caryopsis has reached maximum length when their subsequent disappearance results in the formation of the transparent layer. In section 2.3 of this work, these layers of the pericarp are referred to as the green layer and transparent layer respectively.

Figure 1.3 Transverse section through the centre of a wheat 8.  
caryopsis showing a proposed pathway of assimilate  
transfer ( after Thorne, 1985 )



Key : SE = sieve element  
CC = companion cell  
SC = seed coat

VB = crease vascular bundle  
EC = endosperm cavity  
E = endosperm

The development of the embryo is slower, relative to the endosperm, and it is not until 15-20 days after anthesis that significant development occurs. By this time it can readily be differentiated from the endosperm tissue. A detailed description of the cellular changes accompanying embryo development is given by Smart and O'Brien (1983).

By the time the fertilised caryopsis is about 20 days old the majority of the component tissues, as described in this section, are readily visible to the naked eye. Further development of these tissues does occur, however the most important of these is generally referred to as 'grain filling'. Grain filling is the deposition of storage reserves, principally starch, within the cells of the endosperm. Clearly an understanding of how this process is regulated may lead to improved efficiency of wheat starch production.

### 1.3 Physiological factors related to grain filling

Although the ultimate mechanisms regulating endosperm starch biosynthesis may reside in the biochemical systems responsible for the conversion of assimilate to starch, it is important to consider physiological factors which might influence both the supply of assimilate and its efficiency of uptake into the endosperm cells.

Carbon that is fixed during photosynthesis in the stem, upper leaves and green parts of the ear, moves to the developing caryopsis where most of it is incorporated into starch (Duffus and Cochrane, 1982). However there is no direct symplastic connection between the tissues of the parent plant and those of the endosperm. In wheat (and barley), the caryopsis is supplied by a single vascular bundle which extends along the length of the crease region. Assimilate is believed to reach the endosperm via symplastic movement through the tissues of the crease region and into the apoplast of the endosperm cavity (fig 1.3). In the diagram shown in fig 1.3 the arrows within the cells represent symplastic

movement while the arrows within the cell walls represent apoplastic movement. The curved arrows represent proposed facilitated transport although presently there is no clear evidence to suggest that this, in fact, is the case. Cochrane (1983) has suggested that changes in the chalazal region, occurring during grain filling, may have an effect on the water economy of the developing grain, particularly during the final stages of grain maturation (Cochrane, 1985). Clearly the nature of these crease region tissues may regulate the rate of assimilate transfer into the endosperm with potential consequences for the efficiency of grain filling.

Shannon (1978) has implicated 'sink strength' as an overall regulator of grain filling capacity and defined sink strength (in maize) as 'the efficiency with which the developing kernel imports assimilates and utilises them in the production of starch'. This is a fairly broad definition which can encompass a number of factors. These factors may include the numbers of cells present within the endosperm, the starch storage organelles contained within these cells and the efficiency of the carbohydrate metabolic systems contained within these cells and organelles. Deficiencies in any of these factors may have a detrimental effect on the rate of starch deposition. Alternatively, a controlled excess or increase in any or all of these parameters might lead to increased yields of starch in wheat endosperms.

The starch storage organelles within the endosperm cells are termed amyloplasts and starch is deposited in amyloplasts as organised structures known as granules (May and Buttrose, 1959; Buttrose, 1960). The mature amyloplast is surrounded by a double membrane (Duffus, 1979) which is believed to be physiologically similar to that of the chloroplast. However, at present, there is no clear evidence to suggest that this is entirely the case (Dennis and Miernyk, 1982). Amyloplasts and starch granules are described variously as A- and B- types (Duffus and Cochrane, 1982). While these terms can be applied to the morphology of the granules

(Briarty et al., 1979) it is not clear whether A- and B- type amyloplasts truly exist as separate entities, since this suggests that there are two types of amyloplast. Buttrose (1963) and more recently Parker (1985), have concluded that wheat endosperm B- type granules are found within narrow protrusions extending from the surface of 'A- type amyloplasts'. However there was no evidence to suggest that the B-type granules budded off to form separate amyloplasts. During wheat endosperm development, A-type starch granules have been first observed at around 4 days after anthesis while the B- types did not arise until about 22 days after anthesis (Briarty et al., 1979). In the mature or harvest-ripe endosperm the B- granule contribution to total endosperm starch content has been estimated at 30 per cent (Hughes and Briarty, 1976; Briarty et al., 1979). The number of amyloplasts present in wheat endosperm cells might limit starch biosynthesis and it has been suggested that amyloplast numbers per endosperm are fixed by around 8 days after anthesis (Briarty et al., 1979). Since endosperm cell division in wheat continues up to 20 days after anthesis (section 1.2) then the cells formed post-8 days after anthesis must derive their starch content from amyloplasts that have been passively distributed between mother and daughter cells (P. Cochrane, personal communication). This conclusion may be significant with regard to sink strength, i.e. grain filling capacity in wheat endosperms, since the extent of amyloplast division might indirectly regulate subsequent starch biosynthesis.

The concept of biosynthesis of starch within the amyloplasts of endosperm cells introduces compartmentation as a physiological mechanism of regulation in plant cell metabolism (Dennis and Miernyk, 1982). While amyloplasts have not presently been studied in isolation, there is considerable evidence from studies of chloroplasts to suggest that the double membrane surrounding these photosynthetic plastids is involved in the regulation of carbohydrate metabolism (Heber and Heldt, 1981). However it is not clear whether there is any justification in applying data derived from chloroplasts to the currently unknown situation in

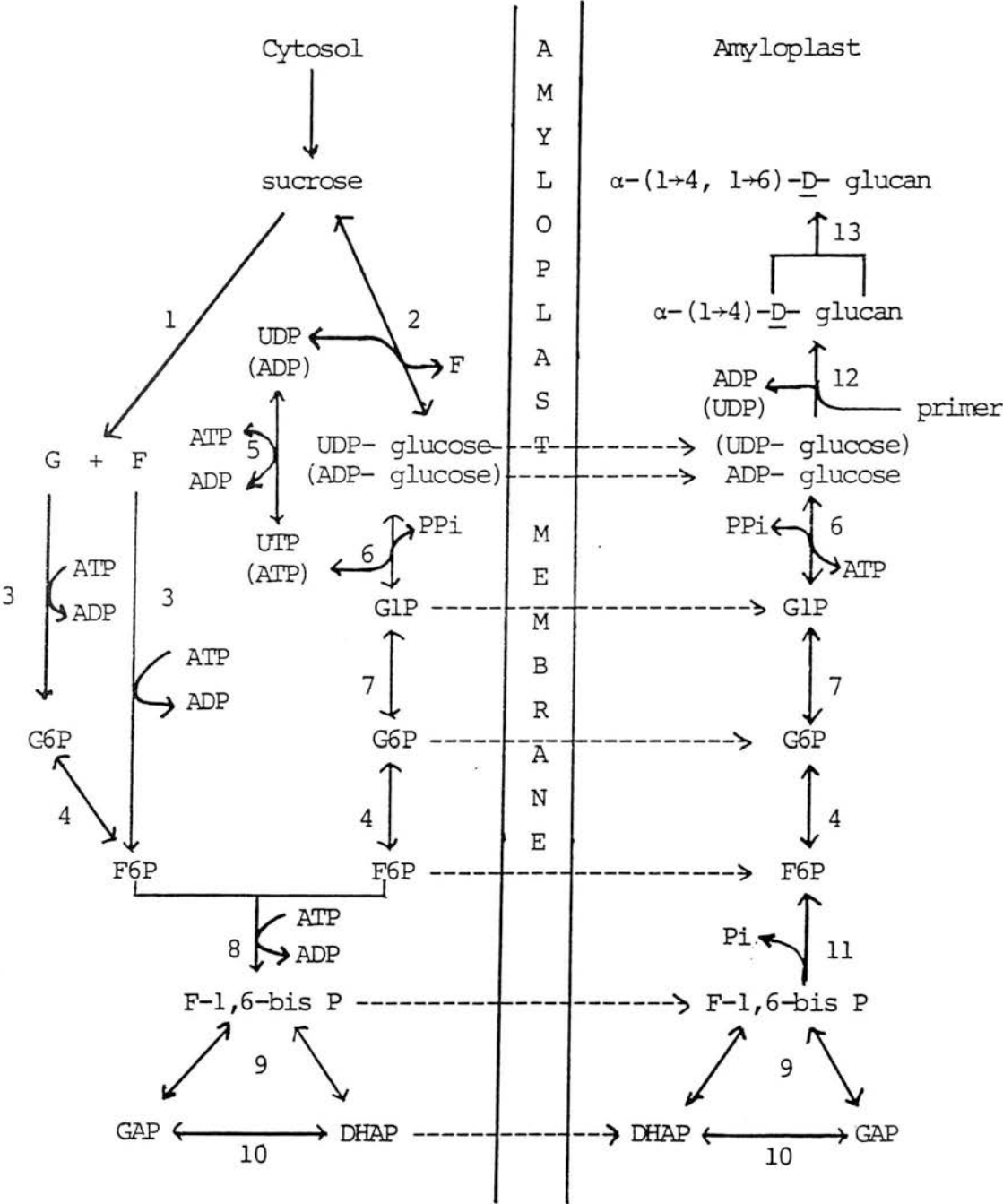
amyloplasts.

Given that the assimilate supply route is open and functional and that endosperm cell and amyloplast numbers respectively are not limiting, then the regulation of endosperm starch biosynthesis may be a function of the efficiency of the metabolic systems concerned with the biosynthesis of starch. These systems may be regulated by the availability of carbohydrate precursors, the effect of these precursors as subsequent substrates and products for the enzyme components of these systems and factors controlling the activity of the individual enzymes. When the phenomenon of compartmentation is taken to encompass all these features then clearly we are dealing with a potentially complex biological system, the regulation of which, presently, is not completely understood.

#### 1.4 Carbohydrate precursors for endosperm starch biosynthesis

Sucrose is the principal transported sugar in wheat and is considered to be the major carbon source for starch biosynthesis in developing endosperms (Duffus and Cochrane, 1982; Preiss, 1982a). However, there is some debate as to whether sucrose reaches the endosperm as sucrose or as its products of invertase catalysed hydrolysis i.e. glucose and fructose. Sakri and Shannon (1975) fed (U- $^{14}\text{C}$ )- fructosyl sucrose to intact wheat ears at 14 days after anthesis. Analysis of the  $^{14}\text{C}$ - label in sucrose, glucose and fructose in subsequent extracts of pericarp and endosperm tissue respectively, showed that the majority of the  $^{14}\text{C}$ - label remained in sucrose, particularly in the endosperm tissue. However it may not be valid to extrapolate the experimental conditions apparent at one developmental age, to those that may apply throughout endosperm development. Additionally, in the absence of simultaneous measurements of enzyme activities, it is not possible to assign estimations of carbon distribution to any particular enzyme system. In order to understand the control mechanisms that may regulate endosperm starch biosynthesis it is necessary to estimate metabolite levels concurrently with determinations of relevant

Figure 1.4 Some of the enzyme catalysed reactions that may be involved in the conversion of sucrose to starch in wheat endosperm cells 13.



2 = sucrose synthase

3 = hexokinase

4 = phosphoglucose isomerase

5 = nucleoside diphosphate kinase

6 = ADP- and UDP- glucose pyrophosphorylases

7 = phosphoglucomutase

8 = phosphofructokinase

9 = aldolase

10 = triose phosphate isomerase

11 = fructose -1,6- biphosphatase

12 = starch synthase

13 = branching enzyme

———— = known reactions in cereal endosperms  
(after Duffus, 1979 )

----- = possible points of transfer between  
cytosol and amyloplast



enzyme activities (Duffus, 1979). Presently this has not been carried out and an important aim of this work is to establish endosperm metabolite levels together with associated enzyme activities.

#### 1.4.1 Carbohydrate metabolism in relation to endosperm starch biosynthesis

Fig 1.4 shows some of the enzyme reactions that could be involved in the conversion of sucrose to starch in wheat endosperms. Although it has been pointed out that there are inherent dangers in the production of comprehensive schemes for starch biosynthesis (Manners, 1974), hypothetical pathways, as shown in fig 1.4, are helpful in that they at least show the potential complexity of the system.

In fig 1.4 the dotted lines indicate possible points in the pathway where metabolites could pass directly into the amyloplast. Clearly if UDP - or ADP - glucose were able to pass directly into the amyloplast and both were substrates for the starch synthase reaction, then the pathway would be quite simple. Such simple schemes have been suggested for starch biosynthesis in rice grains (Lee and Su, 1982) however they do not take into account possible compartmentation effects and in any case their data derives from experimental work carried out on whole rice grains. Such schemes can scarcely be applicable to rice endosperms, let alone for the situation that may exist in wheat endosperms.

The amyloplast membrane may play an important role in the regulation of starch biosynthesis in wheat endosperms. This hypothesis derives mainly from data obtained from spinach chloroplasts. The inner membrane of these organelles exhibits specific permeability for a range of metabolites (Heber and Heldt, 1981). In particular a specific phosphate translocator has been implicated in the facilitated transport of triose phosphates. These triose phosphates are exported from the chloroplast in exchange for

inorganic phosphate (Pi) (Herold, 1980). If an analogous translocator was present in the amyloplast membrane then the most complicated pathway shown in fig 1.4 may prevail i.e. sucrose to dihydroxyacetone phosphate (DHAP) in the cytosol and DHAP to starch in the amyloplast. If this were the case then starch biosynthesis in wheat endosperms may be regulated by Pi export from the amyloplast. Presently there is no evidence to suggest that this is the case, although Pi has been shown to inhibit the activity of ADP- glucose pyrophosphorylase from leaf and bacterial tissues (Preiss, 1982a). This enzyme is also activated by triose phosphates and inhibited by ADP- glucose and inorganic pyrophosphate (PPi) (Preiss and Levi, 1979). In the scheme shown in fig 1.4, ADP- glucose pyrophosphorylase is found both in the cytosol and the amyloplasts. However in the former compartment, the proposed role is the catabolism of ADP- glucose while in the latter case, its role is in the synthesis of ADP- glucose.

Turner and Turner (1975) have suggested that the prominent pyrophosphorylase activity, in relation to sucrose catabolism, was that of UDP- glucose pyrophosphorylase which, in conjunction with UDP- dependent sucrose synthase, was perceived as providing glucose-1-phosphate (G1P) for the ADP- dependent pyrophosphorylase reaction as shown in the amyloplast compartment in fig 1.4. Nucleoside diphosphate kinase activity (reaction 5 in fig 1.4) would provide for the regeneration of UDP for the UDP- dependent sucrose synthase reaction. If this were the case then inorganic pyrophosphatase (PPi) would have to be generated for the cleavage of UDP- glucose (reaction 6). This PPi could come directly from the ADP- glucose pyrophosphorylase reaction in the amyloplast, if a system of PPi translocation existed, and might offer a means of regulating endosperm starch biosynthesis through the translocation of hexose phosphate (cytosolic G1P) in exchange for PPi from the amyloplast. Presently there is no evidence to suggest that this mechanism takes place.

The potential role for ADP- glucose pyrophosphorylase in wheat

endosperm starch biosynthesis has not been properly evaluated since previous work utilised enzyme preparations obtained from wheat flour (Espada, 1962; Tovey and Roberts, 1970). These preparations may have contained residual enzyme activities deriving from chloroplasts (green layer of the pericarp) and also from the embryonic tissues. Wheat leaf ADP- glucose pyrophosphorylase (McDonald and Strobel, 1970) does exhibit the regulatory properties observed in most ADP- glucose pyrophosphorylases from chlorophyllous tissue (Preiss, 1982a). It would be of interest to compare the properties of endosperm and leaf ADP- glucose pyrophosphorylases since an understanding of the relative activities of these enzymes may enable a specific exploitation of a particular function. Clearly if they were both the same enzyme then dual activation might be counter productive i.e. a build up of leaf starch and a consequent reduction in the flow of carbon to the caryopsis.

Endosperm starch biosynthesis may be regulated at the level of sucrose catabolism. Presently we do not know whether the invertase or sucrose synthase pathway (reactions 1 and 2 respectively in fig 1.4) predominates. Energetically the catabolism of sucrose by invertase appears wasteful while UDP (or ADP)- dependent cleavage of sucrose by sucrose synthase conserves the glycosidic bond. Curiously very little work has been carried out on these two enzymes in wheat endosperms and what data there is has been derived from tissues of mixed origin (Meredith and Jenkins, 1976). One of the aims of this investigation is to estimate the significance of either or both of these enzymes to wheat endosperm starch biosynthesis and to measure these activities together with the levels of associated metabolites throughout endosperm development.

### 1.5 Starch synthesis

The principal aim of this current work is to investigate the levels of metabolites and relevant enzyme activities that may be implicated in the regulation of precursor provision for wheat

endosperm starch biosynthesis. Presently these parameters have only been investigated in isolation and, usually, in a variety of tissues of ill-defined origin. Additionally many of the reversible enzyme activities (fig 1.4) have been assayed in the opposite directions to that of their proposed in vivo role. Clearly, this requires re-investigation.

A study of what is essentially intermediate carbohydrate metabolism, leading to endosperm starch biosynthesis, appears to preclude a role for starch synthase and branching enzyme (reactions 12 and 13 in fig 1.4) in the regulation of starch biosynthesis. This preclusion is not intended and what follows is a brief review of the penultimate and final steps of starch synthesis in cereal endosperms. For a more complete discussion the reader is referred to reviews by Banks and Greenwood (1975), Duffus and Cochrane (1982) and Duffus (1984).

The synthesis of linear  $\alpha$ -(1  $\rightarrow$  4)-D-glucan (amylose) and branched  $\alpha$ -(1  $\rightarrow$  4, 1  $\rightarrow$  6)-D-glucan (amylopectin) may require 3 types of enzyme systems i.e. those necessary for (a) primer formation, (b) addition of glucose units by  $\alpha$ -(1  $\rightarrow$  4)- bonds to the primer and (c) introduction of  $\alpha$ -(1  $\rightarrow$  6)- branch points (Banks and Greenwood, 1975).

Primer biosynthesis is presently not clearly understood, although Baxter and Duffus (1973) have suggested that barley endosperm starch phosphorylase may be involved in primer synthesis. However this conclusion was based on levels of unprimed starch phosphorylase activity obtained with partially purified enzyme and these preparations may have been contaminated with primer.

The starch phosphorylase reaction is freely reversible and for a number of years was claimed to be responsible for the GIP-dependent synthesis of amylose. Presently we do not have any data regarding the respective concentrations of GIP and Pi in developing wheat endosperms and it is not possible to eliminate a potential

role for this enzyme in wheat endosperm starch biosynthesis.

The ADP- glucose - dependent starch synthase reaction is considered to be the major enzyme activity concerned with the synthesis of amylose although little is known of the factors which may regulate this reaction (Duffus and Cochrane, 1982). Citrate has been implicated in the activation of some starch synthases (Preiss, 1982b) and this has been linked with flux through respiratory pathways (Duffus and Cochrane, 1982). However, stimulation of starch synthesis by increasing levels of citrate (assuming citrate can enter the amyloplast) might implicate active aerobic respiration in the regulation of starch biosynthesis. This may not be the position in endosperm cells where oxygen could be limiting.

Both ADP- and UDP- glucose may be substrates for the starch synthase reaction and the generation of either of these substrates may regulate the rates of starch synthesis. Clearly if the respective ADP- and UDP- glucose biosynthetic systems are physically separated in endosperm cells, and the amyloplast membrane is impermeable to either of these nucleoside diphosphate sugars, then starch synthesis may depend upon the availability of only one of these potential substrates.

Branching enzyme is not considered, per se, to limit the rate of starch biosynthesis (Duffus and Cochrane, 1982). However the relative activities of starch synthase and branching enzyme seem to affect the character but not the amount of the synthesised starch. High amylose maize endosperm starches are characterised by lowered endosperm branching enzyme activity while the waxy maize endosperm starches (high amylopectin starch) seem to implicate alterations in starch synthase specificities (Nelson, 1980).

It is interesting to note that in mutant strains of maize endosperms where major reductions in levels of endosperm starch have been observed, these reductions have been correlated with deficiencies in endosperm sucrose synthase and ADP- glucose

pyrophosphorylase respectively. Where mutant species are characterised only by alterations in amylose/amylopectin ratios, there were not the same reductions in endosperm starch levels (Nelson, 1980).

Although such mutations have not been observed in wheat endosperms, and indeed may be prevented from occurring by the polyploid constitution of cultivated wheats (Peacock et al, 1981), the data imply that the regulation of wheat endosperm starch biosynthesis might be related to the enzyme systems involved in precursor formation rather than those implicated in the direct synthesis of granular starch.

CHAPTER TWO

2.0 Defined growth characteristics of wheat cv. Sicco

2.1 Introduction

This chapter describes the growth characteristics of wheat cv. Sicco from anthesis to harvest-ripeness. These include accumulation of dry matter and changes in moisture content in both the intact caryopsis and the separated endosperm respectively. The accompanying morphological changes in these tissues, including the embryo are also described.

Table 2.1 (After Zadoks et al., 1974)

General description	Additional remarks
1.0 Milk development	
1.1 caryopsis water ripe	
1.2 early milk	
1.3 medium milk	
1.4 late milk	increase in solids of liquid endosperm notable when crushing the caryopsis between fingers
2.0 Dough development	
2.1 early dough	finger nail impression not held
2.2 soft dough	
2.3 hard dough	finger nail impression held, inflorescence losing chlorophyll
3.0 Ripening	
3.1 caryopsis hard	
3.2 caryopsis hard	difficult to divide by thumb-nail can no longer be dented by thumb-nail
3.3 caryopsis loosening in daytime	
3.4 over-ripe, straw dead and collapsing	

The terminology used to describe these morphological changes derives essentially from the classical work of Percival (1921) who recognized 4 main stages in wheat caryopsis development designated

as milk-ripe, yellow-ripe, ripe and dead-ripe. This work was extended by Saunders (1928) who detailed 5 additional intermediate stages known as late-milk, soft-dough, dough, firm-dough and very-firm. More recently Zadoks et al. (1974) developed a decimal code for computer analysis of the growth stages of cereals and a summary of their system is shown in table 2.1.

None of these systems adequately describes the variety of morphological changes occurring within the tissues of the developing wheat caryopsis, since they rely mainly on a very general description of the life cycle of a single component, namely the endosperm.

Accordingly the present work details a rapid system of morphological ageing, taking into account the significant developmental characteristics of all of the tissues of the wheat caryopsis.

## 2.2 Methods

### 2.2.1 Plant material

Wheat plants (Triticum aestivum L. cv. Sicco) were grown during the summer of 1983 on the Edinburgh School of Agriculture farm at Boghall, Midlothian. Seed was obtained from both the Plant Breeding Institute, Cambridge and the Joseph Nickerson Research Centre, Lincoln. Seeding was carried out on 13 April and the seed rate was 180kg per hectare. Fertilisers were applied prior to drilling at the following rates (kg per hectare): nitrogen, 115; phosphate, 50 and potassium, 50. A further application of nitrogen (50kg per hectare) was made just prior to shoot emergence. Herbicide treatment (Envoy at 2.8 kg per hectare) was carried out on 9 June and 2 fungicide treatments (Tilt at 0.5 litre per hectare) were applied on 24 June and 5 July respectively.



### 2.2.2 Sampling methods

Following ear emergence and subsequent anthesis (see results section 2.3.1) random sampling at 0900h was carried out at 3 and 4 day intervals as detailed below.

At each sampling, 100 main tiller ears were cut below the flag leaf and transported from the field in sealed polythene bags to the laboratory. The basal caryopses (2 per spikelet) together with their respective flowering glumes, were removed from the middle spikelets of each ear. These glumes were then removed and the caryopses placed on moist filter paper. The time period from sampling to isolation of the caryopses was approximately 45 min.

### 2.2.3 Fresh and dry weight determinations

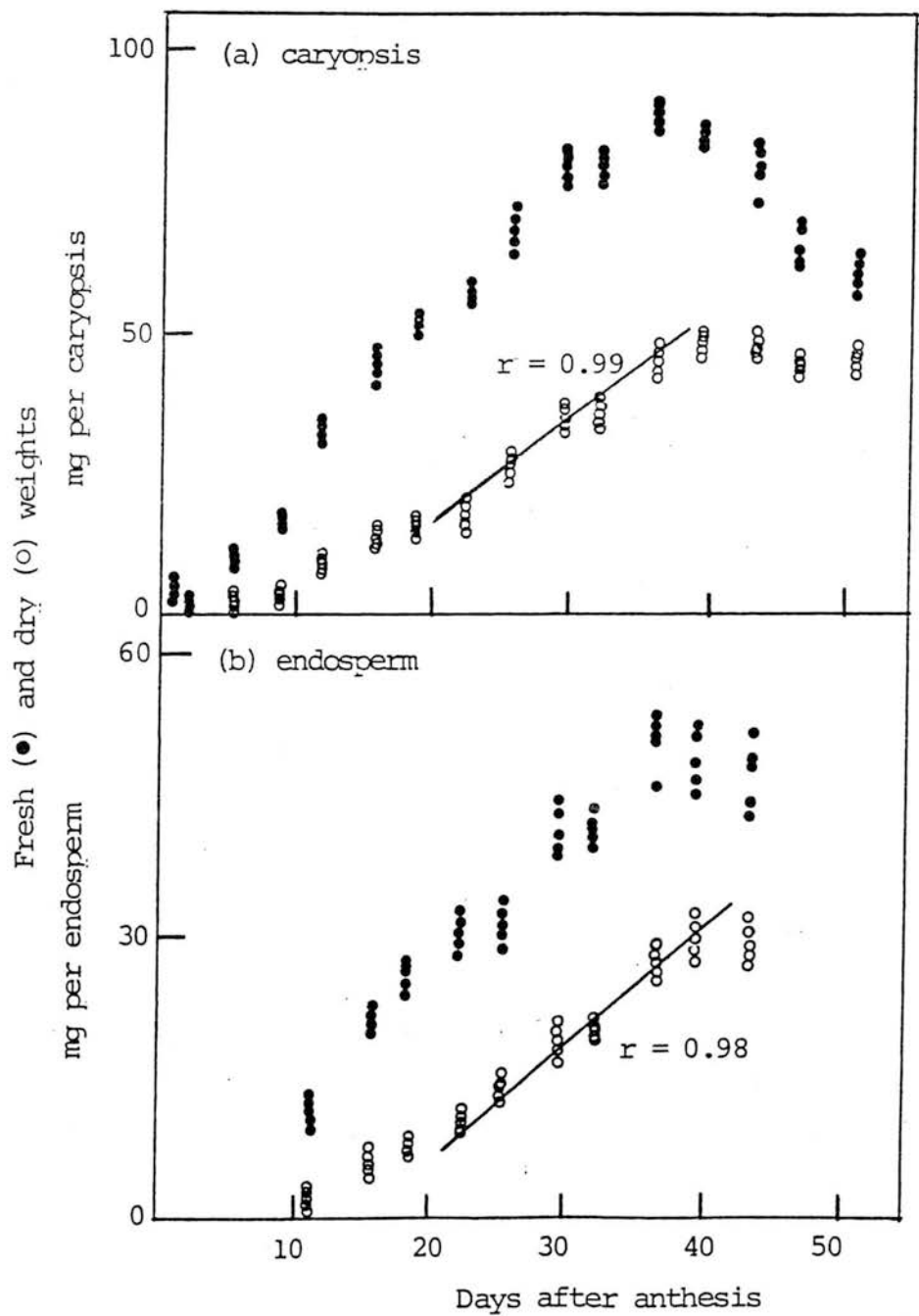
Fifty caryopses were divided into 5 lots of 10 and placed in pre-weighed vials which were then re-weighed prior to placement in a drying oven which was thermostatically controlled at 80°C. Once a steady dry weight had been reached (3-7 days depending upon the age), determinations of fresh weight, dry weight and moisture content of the caryopses were completed.

A further fifty caryopses were dissected to remove pericarp, testa and embryonic tissues and fresh weight, dry weight and moisture content were determined for the remaining endosperms as described for the intact caryopsis.

### 2.2.4 Morphological changes during development

The changes in tissue morphology of the developing caryopses and their component tissues (pericarp, testa, embryo and endosperm) were carefully recorded, together with measurements of length and breadth (caryopsis, embryo and endosperm). These changes and measurements were generally observed without optical aids. Occasionally a dissecting microscope was used to confirm these

Figure 2.1 cv. Sicco, caryopsis and endosperm fresh and dry weight accumulation during development 24.



observations, particularly measurement of the embryo in the early stages of development.

#### 2.2.5 Presentation of data

The graphed data show all of the results obtained from each sampling. Where appropriate, linear trends in the observed data were expressed mathematically by a linear regression line using the method of least squares (Walpole, 1982). The resultant degree of linearity was estimated by the sample correlation coefficient ( $r$ ) and by the sample coefficient of determination ( $r^2$ ). The latter value shows that proportion (expressed as a percentage) of the values of  $Y$  that have a linear relationship with the values of  $X$ . These computations have been taken from Walpole (1982).

The tabulated data show the principal observations in respect of the morphological changes occurring during development. The respective length and breadth measurements for caryopsis, endosperm and embryo were essentially average measurements although the variation within samplings was negligible.

### 2.3 Results

#### 2.3.1 Determination of anthesis

Ear emergence occurred at the beginning of July and all the ears anthesed over a period of 3 days (16-18 July). Anthesis was defined as the emergence of the anthers from within the enclosing flowering glumes. This event had taken place in approximately 75 per cent of all the ears by 17 July and this date was taken as the date of anthesis.

Sampling was continued until 51 days after anthesis (6 September) and the crop was combined at 58 days after anthesis (13 September).

Figure 2.2 cv. Sicco, caryopsis and endosperm moisture content during development

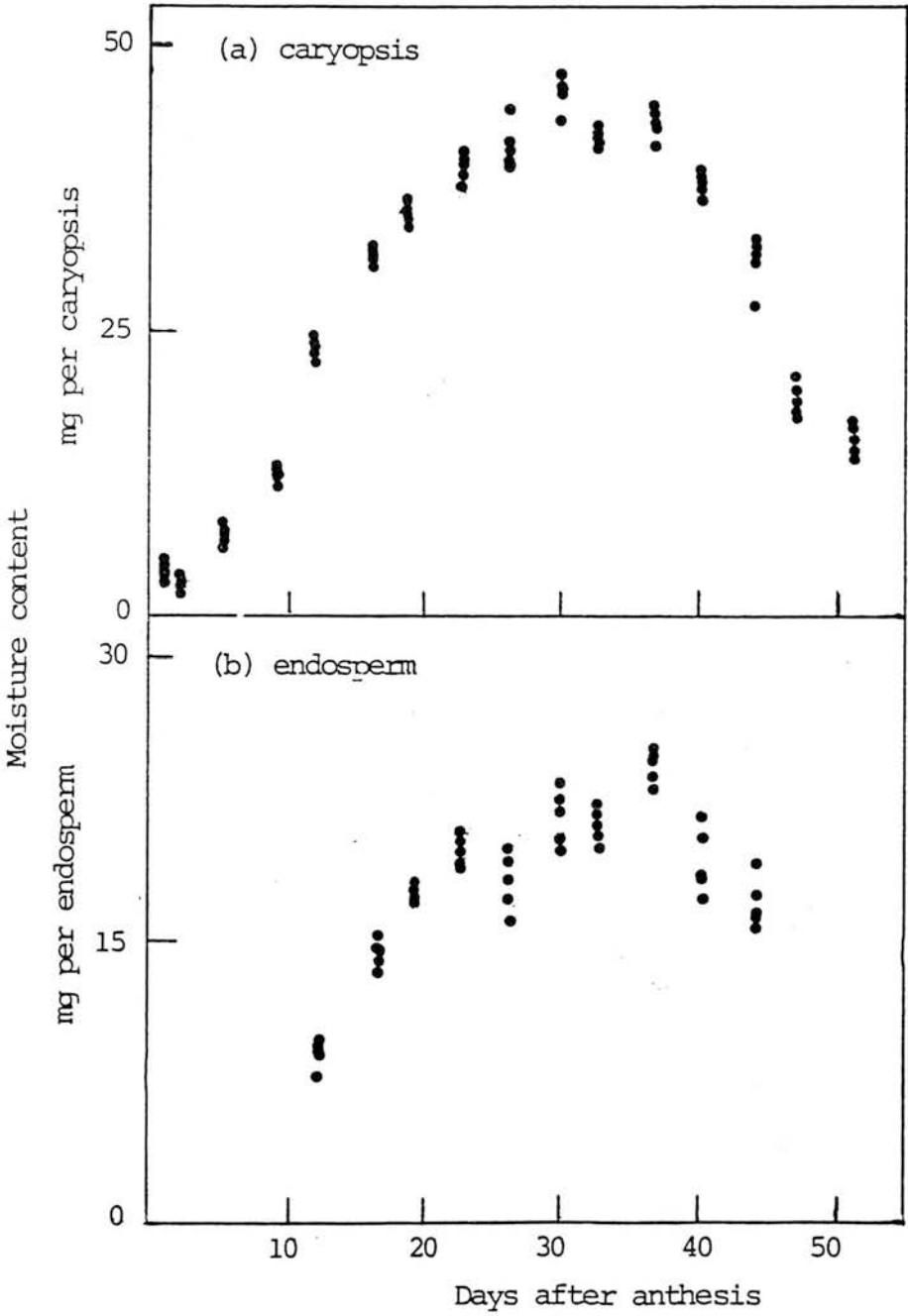


Figure 2.3 cv. Sicco, caryopsis and endosperm percentage  
moisture content during development

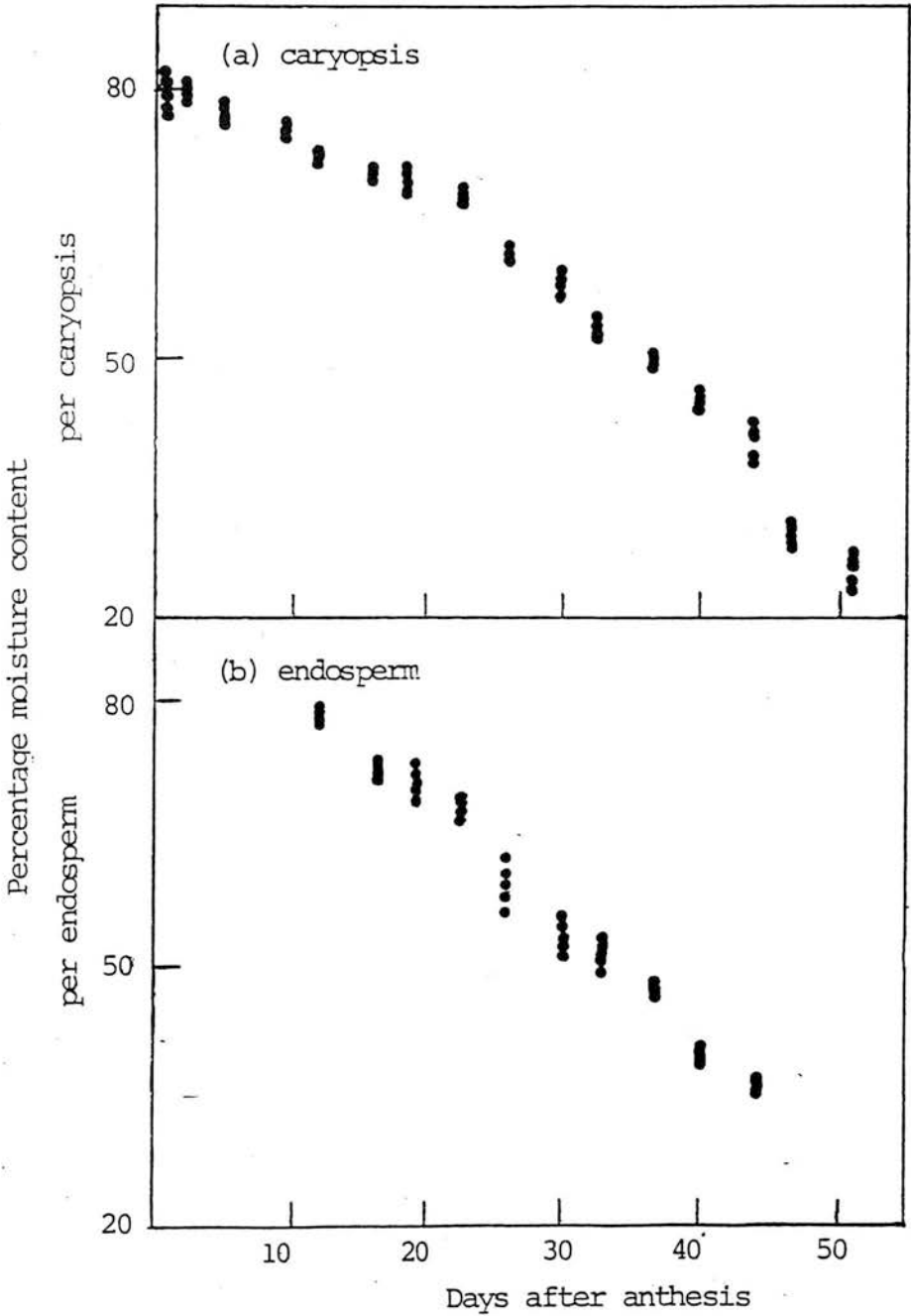
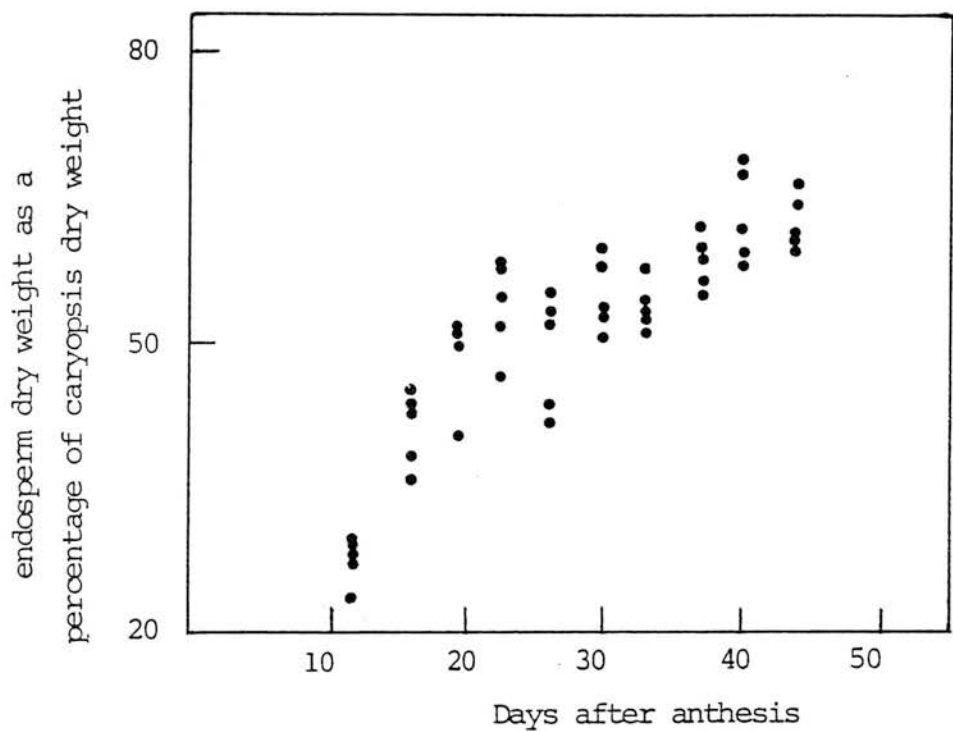


Figure 2.4 cv. Sicco, endosperm dry weight expressed as a percentage of caryopsis dry weight during development 28.



### 2.3.2 Changes in fresh weight, dry weight and moisture content

Following anthesis, caryopsis fresh weight increased steadily to a maximum at 37 days after anthesis (fig 2.1a). During this period, caryopsis dry weight also increased, with the highest rate of increase occurring between 23 and 37 days after anthesis. This rate of increase was linear with respect to time, with the data having a high positive correlation ( $r = 0.99$ ), and averaged 1.8 mg per caryopsis per day. The moisture content of the caryopsis (fig 2.2a) increased rapidly up to 23 days after anthesis but was fairly steady during the subsequent period of rapid dry weight increase. Caryopsis dry weight was essentially constant between 40 and 51 days after anthesis with an observed drop in fresh weight (fig 2.1a) correlating with a rapid loss in moisture content (fig 2.2a). The percentage moisture content of the caryopsis dropped continually throughout development (fig 2.3a).

Whole endosperm tissue could only be dissected between 12 and 44 days after anthesis. Prior to 12 days the endosperm was too fragile for intact removal from the enveloping pericarp tissue, while post - 44 days the pericarp/testa tissue could not be cleanly separated from the endosperm without removal of some of the adjacent endosperm material. These morphological aspects are further dealt with in section 2.3.3.

Endosperm fresh weight increased up to 37 days after anthesis, thereafter showing a slight decrease (fig 2.1b). The dry weight increased continuously between 12 and 40 days after anthesis with the most rapid rate of increase occurring between 23 and 40 days after anthesis. This rate was linear ( $r = 0.98$ ) and was calculated to average approximately 1.2 mg per endosperm per day. The moisture content of the endosperm increased rapidly during the first one-third of dry weight accumulation (fig 2.2b). Thereafter there was only a slight increase as the endosperm continued to increase in dry weight. Post - 40 days after anthesis the endosperm lost moisture. The relative moisture content, expressed as a percentage

Table 2.2 Development of field grown wheat cv. Sicco  
Anthesis 17 July, 1983

Age (days after anthesis)	Appearance of pericarp	Size of caryopsis (mm)	Size of embryo (mm)	Size of endosperm (mm)	Description of endosperm
1	very pale green crease light green	1.0 x 2.0	-	-	-
2	pale green	1.2 x 2.0	-	-	transparent, liquid
5	pale green	3.0 x 2.5	-	-	transparent, liquid
9	pale green crease green	4.0 x 2.5	-	-	transparent, liquid
12	pale green	5.3 x 3.5	-	4.5 x 3.3	translucent, fluid very fragile
16	pale green	6.0 x 3.5	pin tip	5.5 x 3.5	cream, soft moist contents
19	light-medium green	6.0 x 4.0	1.0 x 1.0	5.8 x 4.0	cream, soft moist contents
23	medium green	6.3 x 4.0	1.8 x 1.2	6.2 x 4.0	white, moist becoming sticky



Table 2.2 contd.

Age (days after anthesis)	Appearance of pericarp	Size of caryopsis (mm)	Size of embryo (mm)	Size of endosperm (mm)	Description of endosperm
26	green	6.5 x 5.0	2.5 x 2.0	6.0 x 4.5	milk-white, moist-firming
30	green	6.5 x 5.0	2.5 x 2.0	6.0 x 4.8	milk-white, moist dough
33	green-beginning to yellow	6.5 x 5.0	3.0 x 2.0	6.5 x 4.8	milk-white, soft-doughy
37	yellowing-green	6.5 x 5.0	3.0 x 2.0	6.5 x 5.0	pale-cream, sticky dough
40	yellow, crease still green	6.5 x 5.0	3.0 x 2.0	6.5 x 5.0	cream, drying dough
44	yellow-brown pale green crease	6.3 x 4.5	3.0 x 2.0	6.0 x 4.5	cream, firm, imprint partially remains
* 47	mid-brown, slight wrinkling	6.3 x 4.0	3.0 x 2.0	6.0 x 4.0	cream, dry and rubbery imprint remains
* 51	red-brown	6.0 x 3.5	3.0 x 2.0	-	cream, hard and floury becoming brittle

\* Caryopsis colour: function of testa pigmentation, i.e. pericarp is transparent

Table 2.3 Development of field grown wheat cv. Sicco  
Anthesis 17 July 1983












Age (days after anthesis)	Caryopsis - Shape and Tissue Description			Fused to testa	Description of embryo	Description endosperm
	Shape	Appearance of transparent (TL) and green (GL) layers				
1		TL/GL not clearly differentiated		No	-	(Ovule) clear and liquid
2		TL/GL separation just possible, TL light and fluffy		No	-	Clear and liquid
5		TL/GL separate with difficulty, TL fleshy/moist		No	-	Liquid, no determinate shape
9		TL/GL separate with difficulty, TL fleshy-moist, GL green		No	-	Liquid and fragile
12		TL/GL separate cleanly, TL medium moist		No	-	Can be squeezed out, shallow cheeks
16		TL/GL separate cleanly, TL medium moist		No	cream- pin tip	Cheeks beginning to fill, dorsal area thin
19		TL/GL separate easily, TL medium moist		No	cream- fragile	Cheeks filling, dorsal area thickening
23		TL/GL separate easily, TL medium-thin moist		No	cream- moist and soft	Cheeks filled, dorsal area thickened

Table 2.3 contd.

Age (days after anthesis)	Caryopsis - Shape and Tissue Description			Description of embryo	Description endosperm
	Shape	Appearance of transparent (TL) and green (GL) layers	Fused to testa		
26		TL/GL separate easily, TL becoming thinner	very slightly	Light yellow moist-firming	Cheeks fattening, dorsal area thickened
30	"	TL/GL separate easily, TL still medium, thin, moist	Partial	Light yellow on pale Scutellum, firming	Cheeks and dorsal area well fattened
33	"	TL/GL sticky separation, TL thin, opaque - drying	Partial	Yellow on pale/cream scutellum, firm	Cheeks and dorsal area fattened
37	"	TL/GL sticky to separate, TL thin - drying	Yes	Yellow on pale/cream scutellum firm	"
40		TL/GL sticky to separate, TL thin - transparent, drying	Yes	Yellow on pale scutellum, firm-crisp texture	"
44		TL/GL difficult to separate, TL becoming flakey	Yes	Yellow, firm-crisp	Firm texture, thumb-nail imprint partially remains
47	slight wrinkling	TL/GL difficult to separate, TL transparent/flakey	Yes	"	Dry and rubbery, imprint remains
51	shrunken appearance	"	Yes	Firm/oily in texture	Hard-floury, becoming brittle

of endosperm fresh weight, dropped continuously between 12 and 44 days after anthesis (fig 2.3b)

Fig 2.4 shows the endosperm dry weight data expressed as a percentage of the respective caryopsis dry weights. Initially the endosperm dry weight comprised about 27 per cent of the caryopsis dry weight. This percentage rose to an approximate maximum of 63 per cent between 40 and 44 days after anthesis.

### 2.3.3 Changes in physical dimensions and tissue morphology

Table 2.2 describes the overall colour changes that occurred during development of the Sicco caryopsis together with the changes in size including those of the dissected embryo and endosperm. The general morphological characteristics together with a description of the appearance of the pericarp, embryo and endosperm are shown in table 2.3. There is some overlap in tables 2.2 and 2.3 with respect to the description of the endosperm.

Prior to and immediately after anthesis the caryopsis shape resembled a small triangle referred to by Percival (1921) as a blunt inverted cone. In Sicco, 1 day after anthesis, the caryopsis measured 1mm long and 2mm across its stylar end. During development, the caryopsis gradually increased in size until maximum length and breadth dimensions were reached around 26 days after anthesis. These dimensions remained constant, although volume continued to increase, i.e. the caryopsis became fatter, until around 40 days after anthesis when the caryopsis began to shrink in size. By 51 days after anthesis the caryopsis had a shrunken appearance, and was judged to have reached harvest ripeness.

Changes in the tissues of the pericarp and their relationship to the testa were also observed during development. By 5 days after anthesis it was possible to separate the transparent layer (TL) and the green layer (GL) of the pericarp. At this stage the TL was fleshy and moist and the GL was almost at maximum greenness. This

greenness was dulled by the translucent nature of the TL giving a pale green colour to the intact caryopsis. At 19 days after anthesis the TL was of medium thickness, moist and the green colour was transmitted more strongly, endowing the caryopsis with a light-medium green colour. By 26 days after anthesis the TL was effectively transparent and the overall colour of the pericarp was green. At this stage the TL had become thinner but was still moist and there was a slight adhesion of the GL to the testa. The green colour of the pericarp began to yellow at around 33 days after anthesis and the TL and the GL were becoming difficult to separate cleanly. The TL had become thin and was drying. By 40 days after anthesis the TL was thin, transparent and was drying rapidly. The GL was fused to the testa and these layers were now difficult to separate. The TL and the GL were also difficult to separate and the colour of the caryopsis was yellow although a greenness persisted along the length of the crease. At 47 days after anthesis the TL was transparent and flakey and all the colour had disappeared from the GL. The caryopsis had a mid-brown colour and this was due to a pigmentation of the testa. By 51 days after anthesis the caryopsis had a red-brown colour.

The embryo was first visualised as about the size of a pin tip at around 16 days after anthesis and by 19 days after anthesis it measured 1mm in diameter. At this stage it was pale and fragile and difficult to distinguish, in colour, from the endosperm. By 23 days after anthesis it had attained a size of 1.8 x 1.2mm and was of a moist-soft texture. The embryo continued to develop and by 30 days after anthesis was seen as a well defined tissue having a light yellow colour which contrasted against the pale yellow background of the scutellum. By this time it had a firm texture. Maximum size was reached at 33 days after anthesis but the tissues continued to change in character, eventually developing, at 51 days after anthesis, into a firm, well formed tissue with an oily texture.

For the first 9 days after anthesis the endosperm remained liquid and fragile and could not be dissected intact from the enveloping

pericarp until 12 days after anthesis when it measure 4.5 x 3.3mm. At this stage the contents were translucent, fluid and extremely fragile. The 12 day old endosperm was readily separable from its testa and its shape resembled two shallow cheeks, connected together by a thin dorsal region, rather like two empty panniers slung on either side of the crease region. As development proceeded the cheeks began to fill and by 19 days after anthesis the contents of the endosperm were creamy-moist and a thickening of the dorsal bridge area was detected. By the end of the fourth week of development both the cheeks and the dorsal bridge region were well formed and the contents had reached the milk white - early dough stage. At 33 days after anthesis the endosperm cheeks and dorsal region were well fattened and had a soft-doughy texture. The next 7 days of development saw the endosperm reach its maximum size of 6.5 x 5.0 mm and by 44 days after anthesis the contents had assumed a firm texture. Pressure of the thumb-nail left an imprint which partially remained. There was a slight decrease in endosperm size suggesting a degree of dehydration. By 47 days after anthesis the endosperm was dry and rubbery and readily retained a thumb-nail imprint. At this stage it was extremely difficult to dissect endosperm tissue free of the pericarp-testa while retaining the intact aleurone layer. As harvest-ripeness approached the endosperm was a cream colour and had a floury to hard texture tending to brittleness. At this stage 51 days after anthesis it was not possible to obtain an intact endosperm free of pericarp-testa tissue.

## 2.4 Discussion

### 2.4.1 Determination of anthesis

The determination of the date of anthesis was, to a certain extent subjective since anthesis did not occur at the same time in all ears. However total crop anthesis took place over a period of 3 days and it was considered appropriate to designate the second day

as the date of anthesis, particularly since the majority of the ears had anthesed by the second day.

Chevalier and Lingle (1983) have used a system of ear tagging at anthesis. While this system is adequate for small samplings, it becomes impracticable when large numbers of ears per sample are required. Additionally, ear tagging does not guarantee a uniform rate of development of all the ears tagged at the same time. It can be argued that the same principal applies to a designated date of anthesis, however this system has the advantage of allowing large samplings to be taken at any one time. The benefit of these large samplings was evident in the narrow range of values found within each sampling. The results discussed in later chapters of this thesis were derived from smaller samplings and consequently the range of values at each sampling was extended. The statistical implications of these smaller samplings are discussed in section 3.4.1.

#### 2.4.2 Growth characteristics - grain filling

During the first one-third of both caryopsis and endosperm dry weight accumulations there were significant increases in the respective moisture contents. However when the rates of dry matter accumulation increased, occurring as the caryopsis and endosperm entered into their final two-thirds of dry weight gain, there were apparent reductions in moisture. Sofield et al. (1977a) and Caley (1986) have published similar data for wheat caryopsis and endosperm development respectively, although in the former study the data were derived from plants grown under glasshouse conditions.

The apparent reduction in moisture uptake during the period of rapid dry weight accumulation may be due to a lowered rate of moisture (water) movement into the tissues of the developing caryopsis. Alternatively an increased rate of water efflux from the caryopsis during development might also account for a reduction in



net moisture uptake.

The literature is unclear as to the mechanisms of the overall water economy of developing wheat grains and indeed cereal grains in general (Cochrane, 1983). However there is general agreement that assimilate movement within the phloem occurs in aqueous medium (Jenner, 1982; Lee and Atkey, 1984). The area of disagreement concerns transfer of assimilate to the cells of the developing endosperm. Clearly the endosperm must possess either a means of water removal, or there is a system of disproportionate unloading of solute and solvent (Jenner, 1982). The data in this present work show that the endosperm continued to increase its water content until dry weight accumulation was complete. It is difficult to compare these data with that of other workers (Sofield *et al.*, 1977a; Jenner, 1982; Cook and Oparka, 1983) since their results generally relate to whole caryopses.

In developing rice caryopses (Oparka and Gates, 1981), it has been suggested that the palea and lemma may exert a strong transpirational pull. Consequently these flowering glumes might function in removal of excess water from the developing rice caryopsis. However in wheat a region of xylem discontinuity exists around the point of attachment of the caryopsis to the rachilla (Cochrane, 1983). This region may complicate possible water loss via the flowering glumes of wheat.

Wheat pericarp stomata (Cochrane and Duffus, 1979) might facilitate a means of water loss from the developing caryopsis. Lee and Atkey (1984) investigated this possibility and calculated that these stomata had the potential to account for approximately 18 per cent of the measured rate of water loss. However their data derive from detached caryopses, albeit with palea and lemma attached, and it is difficult to reconcile these results with the situation in the intact plant.

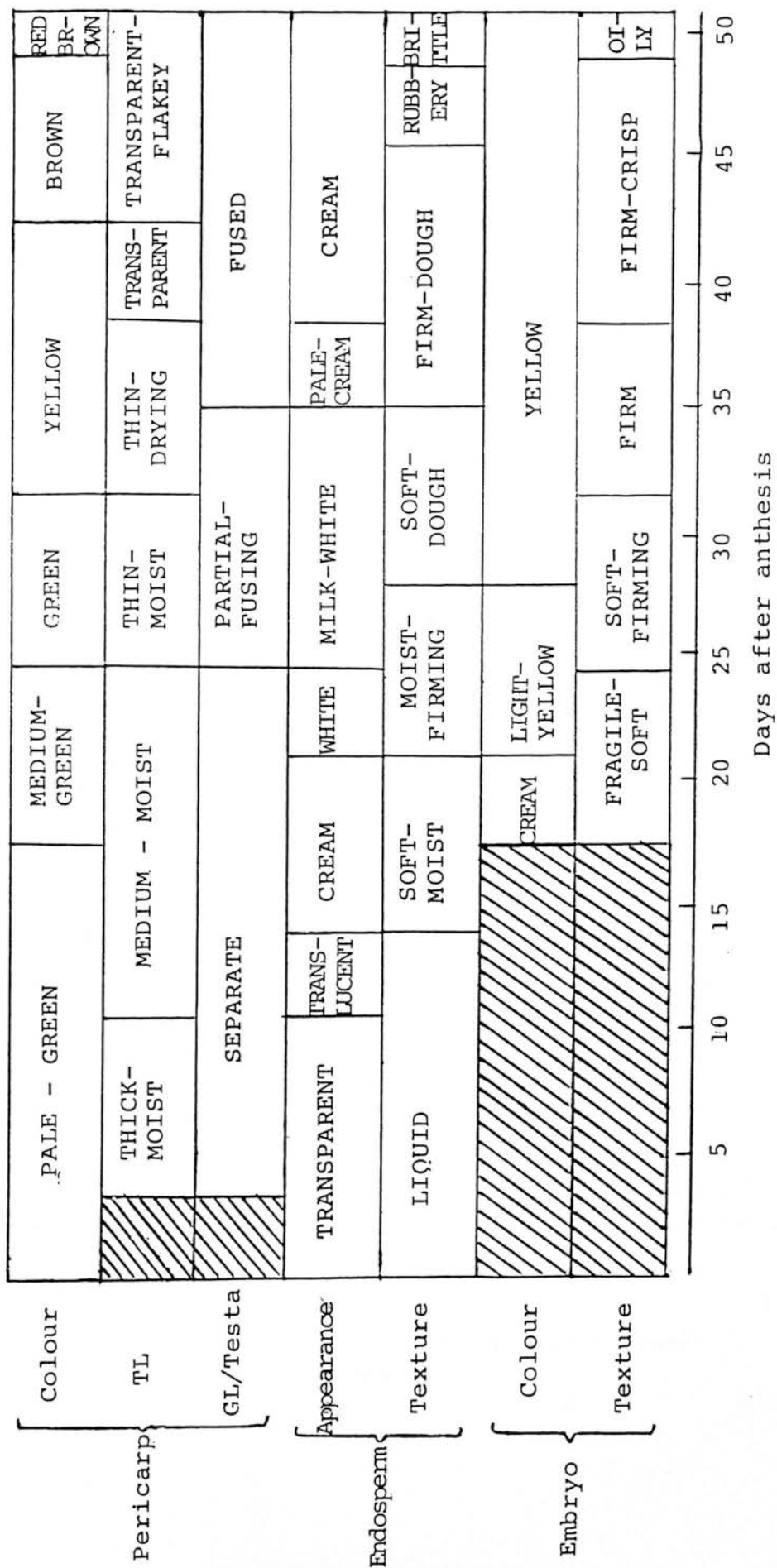
Clearly there are important considerations to be made when



comparing respective moisture losses and dry weight accumulations in developing caryopses and endosperms. There is an obvious problem for the caryopsis, as a whole, in water removal during dry weight accumulation, but this may not be directly comparable to the endosperm. Oparka and Gates (1981) have suggested that assimilate transfer in developing rice endosperms may occur circularly around the endosperm. It was postulated that assimilate moved by active transport from the nucellus into the aleurone cells. Such a mechanism would preclude a build up of water in the endosperm, however it implies the use of biological energy and this may not be consistent with efficient rates of grain filling. Later work (Cook and Oparka, 1983), which involved fluorescein tracer studies in detached caryopses of wheat and barley, suggests a different route for assimilate sic (fluorescein) movement in these cereal caryopses. This movement was believed to occur as a radial spread out of assimilates from the endosperm cavity and this hypothesis was in agreement with the conclusions reached by Watson (1985) who considered a number of possible routes for the movement of  $^{14}\text{C}$ -labelled assimilate into the endosperm cells of barley.

During the early stages of wheat endosperm development there is a clear requirement for water movement into the endosperm, since the endosperm cells are rapidly enlarging but are not yet filled with reserve starch and/or protein (P. Cochrane, personal communication). The data in this present work show that the greatest increase in endosperm length and breadth occurred pre-26 days after anthesis although maximum size was not reached until around 37 days after anthesis. Since the phase of rapid dry weight accumulation did not commence until 23 days after anthesis it is evident that the dimensions of the endosperm, prior to this point in development, must have been maintained by the high percentage moisture content. Post- 23 days after anthesis there was an increasing dry weight contribution with respect to endosperm volume as the percentage moisture continued to fall. Endosperm moisture content only increased by approximately 16 per cent during the latter two-thirds of endosperm dry weight accumulation and most of

Figure 2.5 A summary of the morphological changes occurring during wheat (cv. Sicco) caryopsis and endosperm development



this increase may have occurred by 26 days after anthesis. The results for moisture content at this sampling were inconsistent with the data measured immediately before and after this point.

It is suggested that while flux of water may be critical in regulating caryopsis dry weight accumulation, such a flux might not be necessary for the endosperm per se, particularly during the phase of rapid dry weight accumulation. However in real terms it may not be feasible to consider respective caryopsis and endosperm dry weight accumulations as individual events. This was particularly evident in the latter stages of caryopsis development, where the endosperm contributed approximately 63 per cent of caryopsis dry weight. Clearly, a proper understanding of wheat caryopsis water economy is essential if all of the factors regulating grain filling are to be elucidated. This area of investigation must surely be the subject of future research.

#### 2.4.3 Changes in morphology during caryopsis, endosperm and embryo development

The majority of the data described in later sections of this thesis was obtained from biochemical studies carried out on developing wheat endosperms. These studies had a pre-requisite for ample supplies of fresh plant material which could only be regularly supplied from glasshouse grown crops.

However it was essential that a means of ensuring comparability of tissues be developed. Empirically the length of time between anthesis and harvest-ripeness is dependent upon a number of environmental factors and it is not possible to eliminate variation completely by growing the crops under glasshouse conditions. Fortunately, regardless of the time scale, the wheat caryopsis passes through similar developmental stages at approximately the same relative times (Simmonds and O'Brien, 1981).

These developmental stages as described in section 2.3.3 appear

quite complex, however there were a number of readily defined events which have been incorporated in a box diagram (fig 2.5). The shaded areas represent those events that were difficult to define.

During the course of this present work Rogers and Quatrano (1983) published a system for describing the morphological stages during wheat caryopsis development. Their system was essentially based upon embryo development, as discerned by microscopical examination, but this method is too complex for a rapid ageing system. In addition, carefully controlled growth chamber conditions were used to grow the wheat plants and these conditions are not generally readily available. In any case they do not mimic field conditions.

The system described in this present work, as summarised in fig 2.5, is based on field conditions. It proved to be an extremely rapid, practical method of morphological ageing and the tissues used in the experiments reported in the later sections of this thesis have been age-corrected according to the parameters described in fig 2.5. This ensured that comparable tissues were under investigation at all times. Finally it was considered that the practicalities of this system were such that an ageing error of  $\pm 2$  days might arise. To a certain extent this is a subjective judgement, however it is argued that there is sufficient overlap built into the system to prevent the occurrence of larger errors.

## 2.5 Conclusions

Dry matter accumulation in developing wheat endosperms can readily be linked to distinct morphological changes. For example, the period of rapid dry weight accumulation was consistent with the onset of partial fusing of the testa to the inner layer (GL) of the pericarp, a whitening of the endosperm contents and the appearance of a defined embryo. The slowing of this phase was accompanied by a rapidly drying outer pericarp layer (TL) and a firming-doughy consistency of the endosperm. These and the other morphological events, as summarised in fig 2.5, were closely related to gross

changes in fresh weight, dry weight and moisture content during caryopsis and endosperm development and it is concluded that these latter gross changes can generally be extrapolated from the former morphological events. This ensures that future studies, as reported in the later sections of this thesis, have a sound basis for comparable interpretation. Systems which base conclusions purely on chronological ageing can, at best, only satisfy the experimental conditions present during a specific time course and clearly prevent proper comparison of data derived from different sets of investigations.

## CHAPTER THREE

### 3.0 Determination of some of the metabolites implicated in sucrose-starch conversion in developing wheat endosperms

#### 3.1 Introduction

The rate of dry matter accumulation in wheat endosperms will depend upon the supply of sucrose from the mother plant, the ability of the endosperm to take up sucrose and the efficiency of the metabolic systems that convert sucrose to starch. Since the principal aim of the current work is to investigate some of the biochemical factors which may regulate endosperm starch biosynthesis, the present section will be concerned with an estimation of various metabolites that are both substrates and products of the wheat endosperm enzyme systems implicated in sucrose-starch conversion.

Up till the present time, only very limited information on wheat endosperm metabolites has been available and much of this relates exclusively to determinations of sucrose (Jenner and Rathjen, 1972; Jenner and Rathjen, 1975; Jenner and Rathjen 1977). Additionally the previous work was carried out on either whole grains or partially dissected endosperms, sampled at only a few stages during development.

Jenner (1968) obtained a variety of data on the composition of soluble nucleotides from exclusively wheat endosperm material, but only at 2 stages in development. Time course studies for UMP, UDP, UTP, UDP-glucose and the adenine equivalents were carried out on whole grains.

A more recent investigation (Kumar and Singh, 1981) examined free sugars and starch content in developing wheat grains from 4 wheat varieties. This work is of very limited value since no attempt was

made to separate the various tissues of the wheat caryopsis and, other than sucrose, there was no specificity of sugar analysis, with the exception of reducing power.

The current work describes the levels of sucrose and some of the intermediate metabolites associated with sucrose-starch conversion in developing wheat endosperms. The levels of these metabolites will be discussed in relation to the enzyme systems associated with their conversion. Later sections of this thesis are devoted to a study of these enzyme systems. Since no such integrated approach presently exists, it is hoped that these studies will enhance our knowledge of how wheat endosperm starch biosynthesis may be regulated.

### 3.2 Methods

#### 3.2.1 Plant material

Wheat seed (Triticum aestivum cv. Sicco) was obtained as previously described (section 2.2.1) and sown into plastic pots (180mm diameter) containing Levington's compost. Six plants per pot were maintained under glass at the Crop Production Glasshouse Unit, Bush Estate, Midlothian. Sowing was carried out at fortnightly intervals throughout the year and the young plants were transferred, at about the time of anthesis, to the Department of Agricultural Biochemistry, The Edinburgh School of Agriculture, West Mains Road, Edinburgh. The plants continued to be maintained under glass where natural daylight was extended to 20h using mercury vapour lamps (400W). The average day and night temperature was thermostatically controlled at 18°C by the use of fan heaters and ventilation fans. The plants were watered daily and fed three times per week with an aqueous solution composed of (mM):  $\text{KNO}_3$ , 2.0;  $(\text{NH}_4)_2\text{HPO}_4$ , 0.25 and  $\text{NH}_4\text{NO}_3$ , 1.0. Insect and fungal infections were minimised by occasional spraying with Pirimor (ICI) and Milgo (ICI).

A set of 24 pots, containing 6 plants each, were selected for

analyses of metabolites during endosperm development. Each plant produced, on average, 4-6 tillers and ears deriving from the tallest tillers on each plant were randomly sampled at suitable intervals throughout the sampling period. The ears were removed by cutting below the flag leaf and the cut ears removed to the laboratory for analysis. Ears (24) were separated into 4 lots of 6 and the basal caryopses from the middle spikelets removed from each lot and placed on moist filter papers (1 per lot). Sample endosperms from each lot were quickly aged according to the system described in section 2.4.3 and a number (8 to 34 depending upon stage of development) were dissected free of embryonic and pericarp/testa tissue and prepared for metabolite analysis.

### 3.2.2 Reagents

Bovine serum albumin (fraction V and essentially fatty acid free), glucose-6-phosphate dehydrogenase (type IX), inulin, phosphoglucomutase and phosphoglucose isomerase (type X) were obtained from Sigma Chemical Co. Ltd., Poole, Dorset.

Cocksfoot fructan was a gift from Dr W Dewar, The Poultry Research Centre, Roslin, Midlothian.

All other reagents were of analytical grade or of the highest purity available.

### 3.2.3 Metabolite analysis

#### 3.2.3.1 Preparation of endosperm extracts

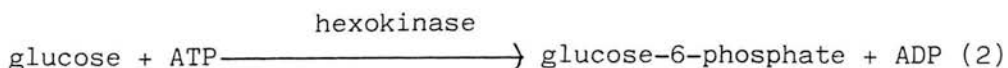
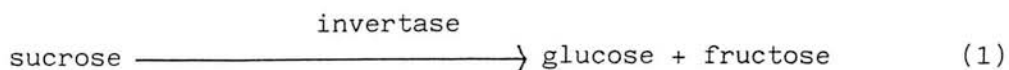
Samples of dissected endosperms were homogenised on ice in 1M perchloric acid (1.03ml) for 5 min using a hand-held Potter-type all-glass homogeniser. Chilled distilled water (1.47ml) was then added and the mixture further homogenised on ice for 10 min. Following homogenisation the sample was quantitatively transferred to a centrifuge tube using further aliquots of chilled distilled

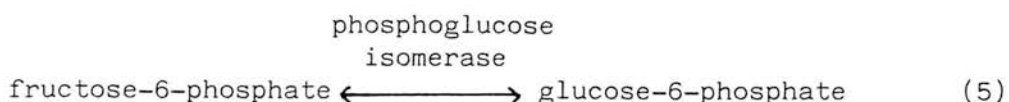
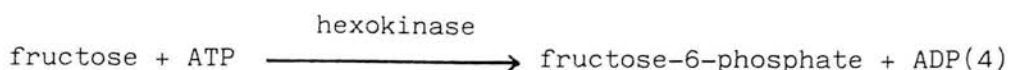
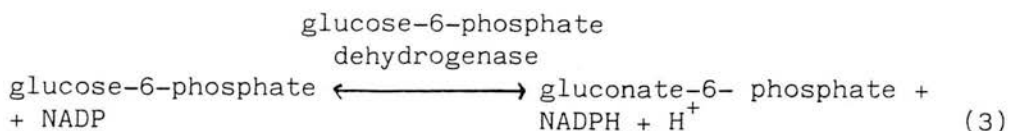


water (2 x 1ml) and the complete homogenate centrifuged for 10 min at  $< 4^{\circ}\text{C}$  and 3 500g ( $r_{\text{av}} = 10\text{cm}$ ) in an MSE chillspin centrifuge. The resulting supernatant was retained on ice and the pellet resuspended in 1M perchloric acid (0.34ml) and mixed on ice for 5 min. Chilled distilled water (1.17ml) was added and, following an additional period of mixing on ice (10 min), the resuspended pellet was centrifuged as described for the initial homogenate. Following centrifugation, the second supernatant was added to the first and the final pellet retained on ice. The combined supernatants were then neutralised by first adding 0.3ml of  $\text{KH}_2\text{PO}_4$  (1M) and then KOH (5M) to pH 7.0 (P. Keeling, personal communication). The neutralised supernatant was then recentrifuged as previously described and the supernatant (PCA extract) measured for volume and then stored at  $-20^{\circ}\text{C}$ . The pellet was discarded. The final pellet, deriving from the first and second perchloric acid extractions and previously retained on ice, was mixed with 1M NaOH (3ml) and heated for 10 min in a water bath, thermostatically controlled at  $65^{\circ}\text{C}$  (Passonneau *et al.*, 1978). The dissolved pellet was then cooled on ice and centrifuged as for the PCA extract. The supernatant was measured for volume and then stored at  $-20^{\circ}\text{C}$ . The pellet was discarded.

### 3.2.3.2 Determinations of sugars and sugar phosphates

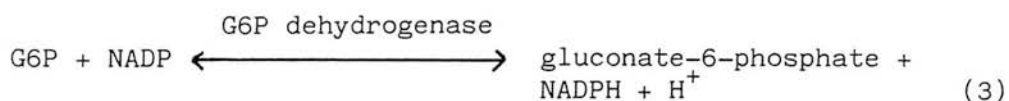
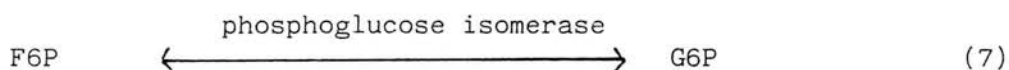
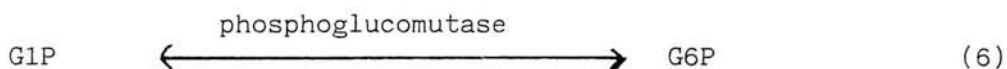
Sucrose, glucose and fructose were determined using test combination kits supplied by Boehringer Mannheim and essentially by the enzymic procedures described in their instruction manual (Boehringer Mannheim, 1983). Their methods utilise the following enzymic reactions:





For the analysis of sucrose, reactions 1,2 and 3 were coupled. Glucose was determined by combining reactions 2 and 3, while fructose was measured by coupling reactions 4,5 and 3.

Since the test combination kits were designed primarily for food analysis, they did not take into account endogenous levels of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) that might be present in the PCA extracts. In order to estimate these metabolites, pure enzyme preparations were obtained from Sigma Chemical Company in order to catalyse the following reactions:



Thus G1P was estimated by coupling reactions 6 and 3, F6P was measured by combining reactions 7 and 3 and G6P was analysed by reaction 3 on its own.

For the analysis of these sugar phosphates the following solutions were required: solution 1 (sln 1) was composed of 152 mM triethanolamine buffer at pH 7.6 containing NADP (3.04mM) and  $\text{MgSO}_4$  (30.4 mM); solution 2 (sln2) contained G6P dehydrogenase (EC

1.1.1.49), 500 units dissolved in 50mM triethanolamine buffer (3.45ml) at pH 7.6 containing 0.1% w/v bovine serum albumin (BSA); solution 3 (sln 3) contained phosphoglucomutase (EC 2.7.5.1), 875 units dissolved in 50mM triethanolamine buffer (1.75ml) at pH 7.6 containing 0.1% w/v BSA; solution 4 (soln 4) contained phosphoglucose isomerase (EC 5.3.1.9), 1000 units in 50mM triethanolamine buffer (2ml) at pH 7.6 containing 0.1% w/v BSA.

All determinations, including mixing of reagents, were carried out in Sarstedt 10 x 10 x 45mm acrylic cuvettes suitable for the spectrophotometric analysis of NADPH at 340 nm. Absorbances at that wavelength were measured at 25°C in a Pye Unicam SP8/100 UV/Vis spectrophotometer.

For the determination of G6P and G1P an aliquot (0.1ml) of the PCA extract was pre-incubated for 5 min at 25°C followed by the addition of sln 1 (1.0ml) and distilled water (1.9ml). The contents were thoroughly mixed and allowed to stand for approximately 3 min when the absorbance at 340nm was noted. The reaction was initiated by the addition of sln 2 (0.02ml) and the absorbance was read at 340nm after 15 min or as soon as the reaction became steady. Sln 3 (0.02ml) was then added and the absorbance at 340nm re-read after a further 15 min or as soon as the reaction became steady. Suitable water blanks were incorporated as were calibration standards for both G6P and G1P in the range 0-100  $\mu$  M.

F6P was determined in a similar manner to G6P and G1P except that sln 4 (0.02ml) was added instead of sln 3. Water blanks and calibration standards in the range 0-100  $\mu$  M were incorporated in the analysis.

Recovery percentages for sucrose, glucose, fructose, G1P, G6P and F6P were calculated by the addition of known quantities of these metabolites to dissected endosperms, prior to preparation of PCA extracts as described in section 3.2.3.1. These metabolites were

added in 0.1ml aliquots at the following concentrations (mM): sucrose, 95; glucose, 7; fructose, 7; G1P, 3.5; G6P, 5 and F6P, 5 to dissected endosperms (10) with a morphological age of 30 days after anthesis. Duplicate controls without the addition of exogenous metabolites were also prepared for PCA extraction. Following preparation of the respective PCA extracts the sugars and sugar phosphates were determined by the enzymic procedures as detailed above.

Controls to check for possible perchloric acid hydrolysis of endosperm fructans were carried out by subjecting solutions of fructan (2.5% w/v) and inulin (2.5% w/v) to perchloric acid extraction. Aliquots (0.1ml) of these solutions were extracted as described in section 3.2.3.1 and the respective PCA extracts estimated for fructose as described in this section. Blanks were prepared by substituting distilled water for perchloric acid.

#### 3.2.3.3 Ethanolic extracts

The efficiency of the perchloric acid extraction of endosperm tissue (section 3.2.3.1) was compared with a hot ethanol extraction in the following manner.

Endosperms (20) with a morphological age of 30 days after anthesis were divided into 2 lots of 10 endosperms each. The first lot was subjected to perchloric acid extraction as described in section 3.2.3.1, while the second lot was homogenised for 10 min in 80 per cent v/v ethanol (2ml) maintained at 85°C in a thermostatically-controlled hot water bath. Following homogenisation, the homogenate was allowed to stand for a further 20 min at 85°C and was then quantitatively washed out with hot 80 per cent v/v ethanol (3 x 1ml). The mixture was then centrifuged for 15 min at ambient temperatures and 5 000g ( $r_{av} = 11.2\text{cm}$ ) in an MSE High Speed 18 centrifuge. The resultant supernatant was retained on ice and the pellet resuspended in hot 80 per cent v/v

ethanol (3ml) and re-homogenised as described for the initial homogenisation. The suspension was centrifuged as described above and the supernatant combined with the first, the total volume measured and the combined supernatants were then stored at  $-20^{\circ}\text{C}$ . The final pellet was treated with NaOH as described in section 3.2.3.1.

These extracts (PCA and ethanolic) were subsequently analysed as described in section 3.2.3.2.

#### 3.2.3.4 Determinations of nucleoside diphosphate sugars and nucleoside di- and triphosphates

PCA extracts as described in section 3.2.3.1 were analysed at ICI, Runcorn using High Performance Liquid Chromatography (HPLC) comprising: Waters Wisp 710B Automatic Injector; Du Pont Instruments Column Compartment housing a Partisil 10 SAX Anion exchange column (25 x 0.46cm) with a 5cm guard column of the same material; Du Pont Instruments 870 pump module; Du Pont Instruments 8800 Gradient Controller and Trivector Data system incorporating VDU, Trilab 2000 Multi-channel Chromatography Data System and a Trivector 2012 Printer Plotter. The temperature of the column was maintained at  $35^{\circ}\text{C}$  within the column compartment. Eluate was monitored at 280nm by a Kratos Spectroflow 773 detector. A binary gradient system comprising solvent A (2mM  $\text{KH}_2\text{PO}_4$  at pH 3.3) and solvent B (600 mM  $\text{KH}_2\text{PO}_4$  and 1.1M KCl at pH 3.3) was used as the mobile phase. The running conditions were 100 per cent A for 15 min, 0-100 per cent B for 25 min and 100 per cent B for 10 min. The flow rate was constant at 1ml per min. Total run time, excluding equilibration period, was 50 min. The solvents were filtered through a 0.45 micron filter (Millipore) and degassed (solvent degasser ERMA optical Works Ltd, Japan) prior to pumping. The sample injection volume was 0.05ml.

Two sets of calibration standards were run for each metabolite



under analysis. The concentration ranges were 0 - 20  $\mu$  M and 0 - 100  $\mu$  M. Standards were made up in distilled water just prior to injection and were run for each fresh batch of solvent.

Recovery percentages were calculated by the addition of 30  $\mu$  l of the respective standard at a concentration of 16mM to samples of dissected endosperms (15) with a morphological age of 35 days after anthesis. These samples together with duplicate samples, without added standards, were extracted as described in section 3.2.3.1. Recovery percentages were calculated for each batch of samples.

The stability of the nucleoside diphosphate sugars and the nucleoside di- and triphosphates to extraction by perchloric acid was investigated using various concentrations (0-3M) of perchloric acid in the extraction procedure (section 3.2.3.1) but in the absence of any endosperm material. The resulting neutralised PCA extracts were chromatographed as described in this section and the presence of any breakdown products determined.

#### 3.2.3.5 Measurement of soluble protein

Soluble protein was measured essentially according to the method of Lowry et al (1951). BSA (50mg) has dissolved in distilled water (4ml) and made up to 5ml in a volumetric flask. This stock solution was diluted 100 times to give a final BSA concentration of 100  $\mu$  g per ml. From this stock solution a series of standards were prepared in the range 0-100  $\mu$  g per ml. The assay solutions (sln) were composed of : sln 1,  $\text{Na}_2\text{CO}_3$  (0.94M) containing NaOH (0.50M) sln 2, potassium sodium tartrate (0.07M); sln 3,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.03M) and sln 4, Folin-Ciocalteu diluted 11 times with distilled water. All assay solutions were stored at 4°C in the refrigerator and the diluted Folin reagent was made up just before use. For determination of protein sln 2 (2.5ml) was mixed with sln 3 (2.5ml) and sln 1 (50ml) was added. Aliquots (1ml) of this mixture were added to assay samples or standards (1ml) respectively, with the former being suitably diluted. Following mixing and a 10 min

Figure 3.1 Amounts of sucrose, glucose and fructose during endosperm development

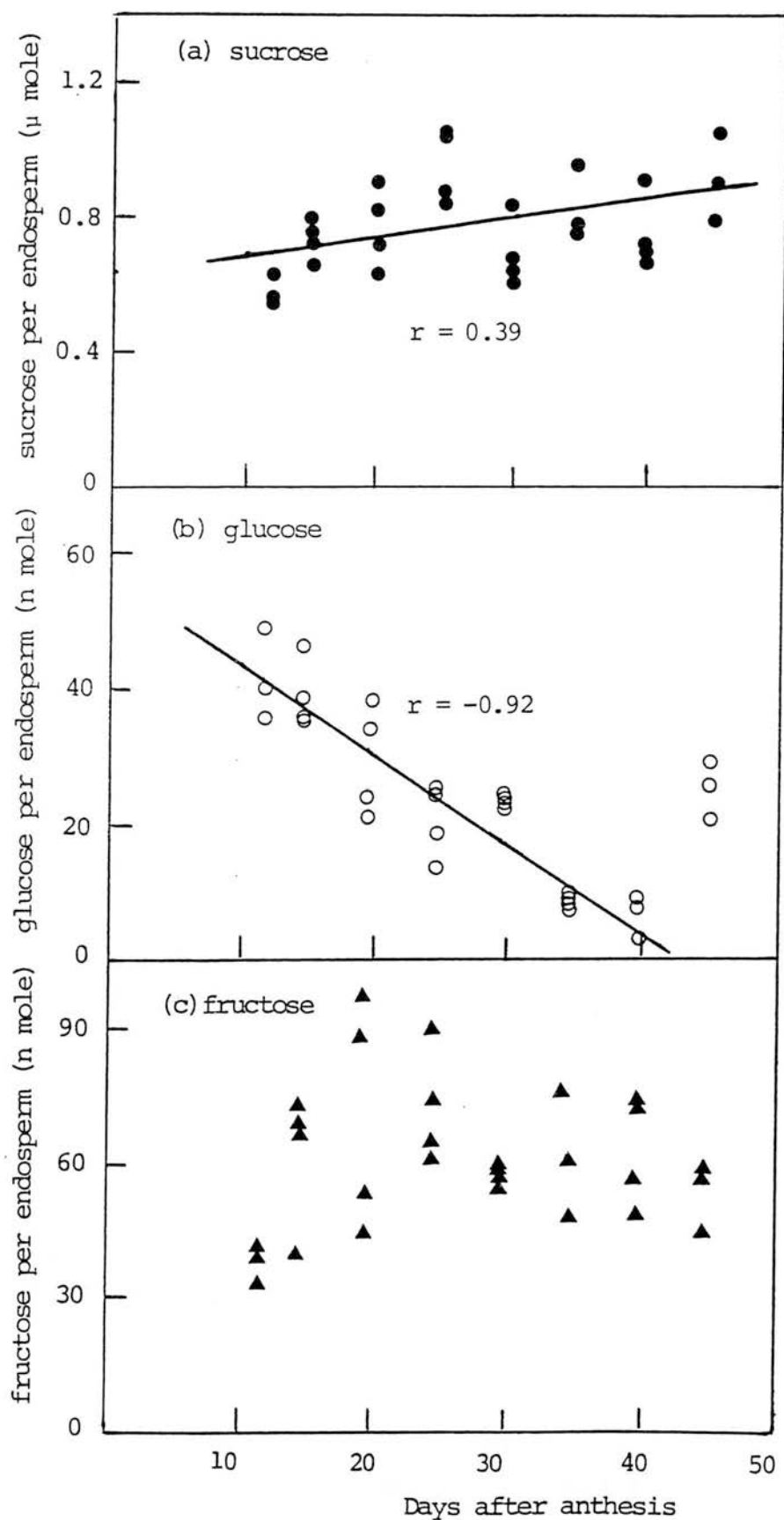


Figure 3.2 Amounts of GlP, G6P and F6P during endosperm development

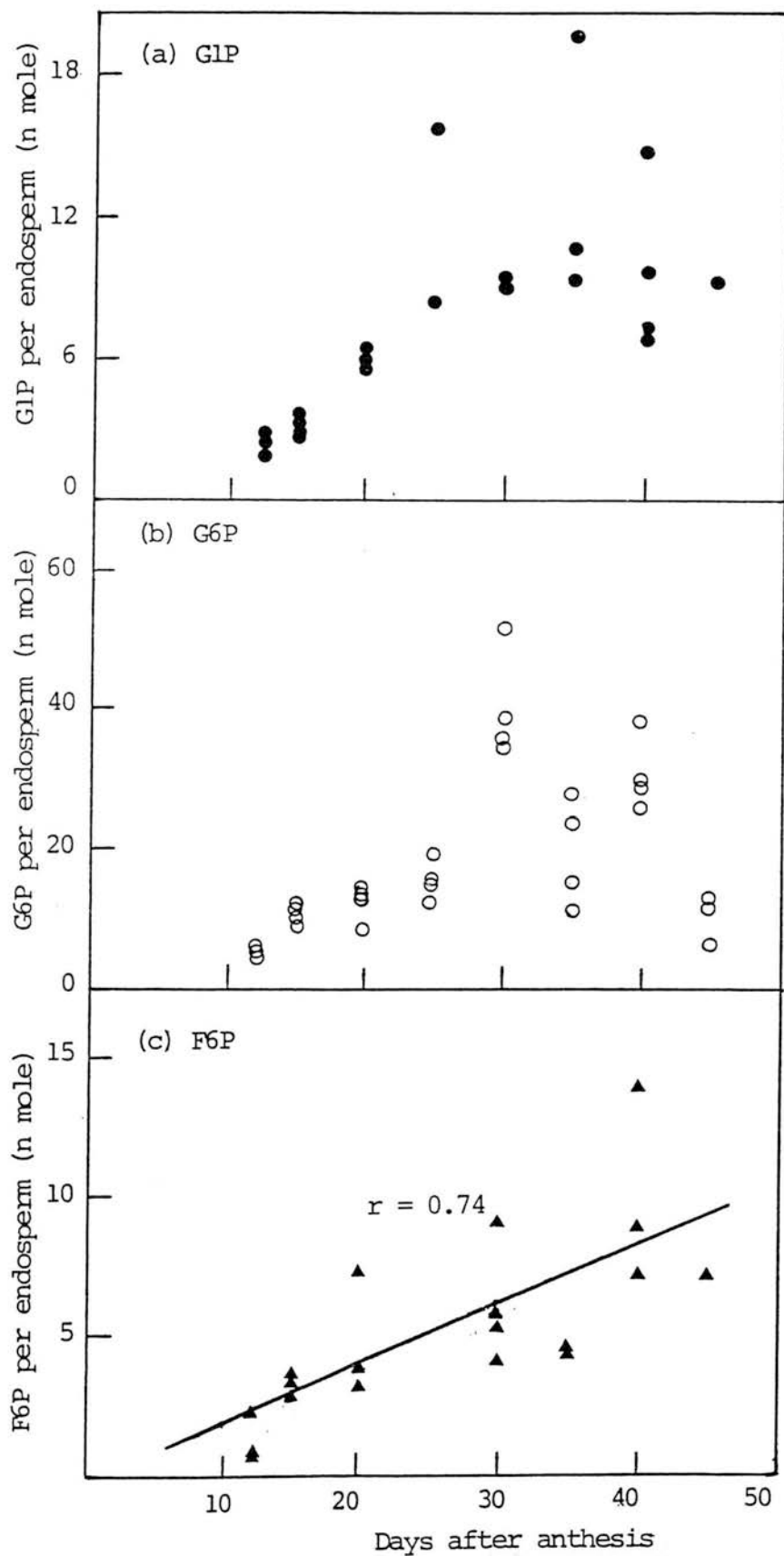
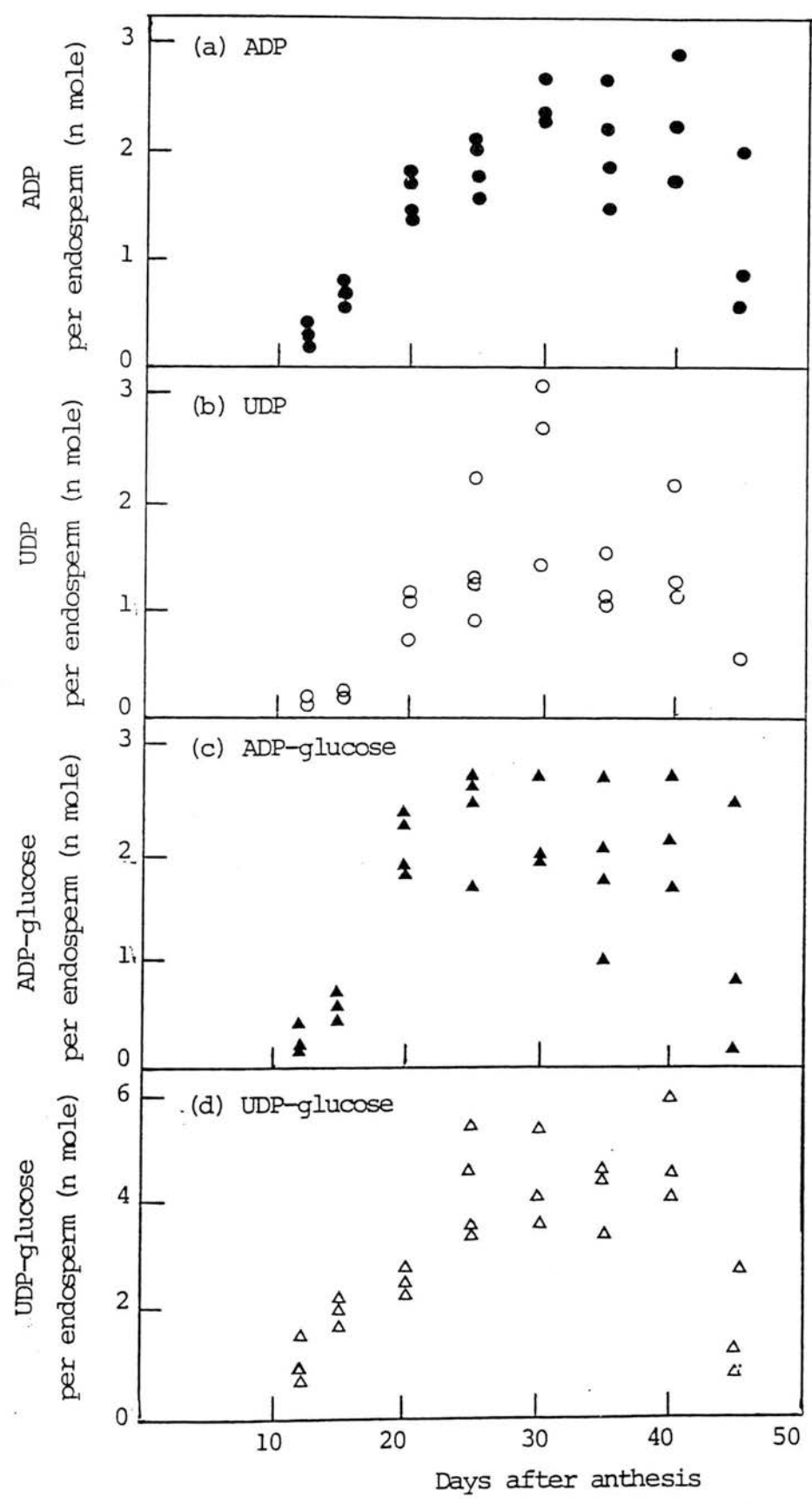




Figure 3.3 Amounts of ADP, UDP, ADP-glucose and UDP-glucose during endosperm development



incubation at room temperature, sln 4 (3ml) was rapidly added followed by an immediate and thorough mixing. After a further 30 min incubation at room temperature the optical densities were measured using Sarstedt 10 x 10 x 45 mm acrylic cuvettes at 650nm in a Pye Unicam SP 8/100 UV/Vis spectrophotometer. A calibration curve was prepared from the standards and the concentration of protein in the assay samples determined from this curve.

#### 3.2.3.6 Analysis of data

Where appropriate, data were analysed as previously described in section 2.2.5.

### 3.3 Results

#### 3.3.1. Metabolite determinations

The results from the various metabolite determinations during endosperm development are shown in figs 3.1, 3.2 and 3.3. Each point represents the respective individual determination from each endosperm extract. The amounts have been expressed per endosperm.

The levels of sucrose throughout the sampling period were estimated in  $\mu$  moles per endosperm while the amounts of the other metabolites were calculated in n moles per endosperm. In all cases the results are shown uncorrected with respect to per cent recovery. The calculated recovery percentages are given in table 3.1.

Table 3.1 Recovery percentages

Metabolite	% recovery
sucrose	115
glucose	88
fructose	60
glucose-1-phosphate	66
glucose-6-phosphate	98
fructose-6-phosphate	93
ADP	70
UDP	80
ADP-glucose	82
UDP-glucose	80
ATP	87
UTP	84

These data indicate that, with the exception of sucrose, the observed amounts present were underestimated.

Sucrose levels (fig 3.1a) rose very slightly throughout endosperm development, the measured values having a slight positive correlation ( $r = 0.39$ ) i.e. approximately 15 per cent of the values increased linearly with respect to development time (see section 2.2.5).

In contrast the amount of glucose per endosperm (fig 3.1b) declined linearly from 12 to 40 days after anthesis with a high negative correlation ( $r = -0.92$ ). An apparent rise was recorded for the 3 samples analysed at 45 days after anthesis. Estimations of fructose during endosperm development (fig 3.1c) showed a pattern quite different from that of glucose. While measurements at 12 days after anthesis were similar to those recorded for glucose, the levels then rose, with maximum values recorded at around 25 days after anthesis. At this point the mean amount for fructose was approximately 4 times greater than that for glucose and around 13 times less than that for sucrose. Thereafter a slight decline was

observed during the remaining period of endosperm development. The ratio of amounts of fructose to glucose rose consistently throughout endosperm development with the value approaching 10 at 40 days after anthesis. The same parameter for fructose to sucrose was essentially constant at a value of 0.08 during the entire period of endosperm development.

The possibility that fructose values may have been overestimated due to hydrolysis of fructans was investigated. However there was no evidence of hydrolytic release of fructose from the sample of Cocksfoot fructan, although the commercial inulin yielded some fructose. This was found to be 3 per cent of that available for hydrolysis.

The amounts of G1P, G6P and F6P present in endosperms are shown in fig 3.2. The levels of the 3 hexose phosphates increased during the first 25 days after anthesis with the rate of increase greatest for G1P. The recovery rate (table 3.1) for this hexose phosphate was low, while G6P and F6P had substantially higher recovery rates. Determinations of G1P during the later stages of endosperm development suggested that the amounts were tending towards a constant value, although there was an apparent high value for one of the samples taken at 35 days after anthesis. This value was almost double that of the other 2 values in the sample set and may be erroneous. G6P per endosperm rose to a maximum at 30 days after anthesis, before declining irregularly, until at 45 days after anthesis the values approximated to those determined at the beginning of endosperm development. F6P per endosperm tended to rise throughout the entire sample period and this rise showed a moderate positive correlation ( $r = 0.74$ ) with 55 per cent of all determinations having a linear relationship with developmental time. However these data do not include a substantial number of samples in which F6P was not detected.

The amounts of ADP, UDP, ADP-glucose and UDP-glucose during

endosperm development are shown in fig 3.3.

In general, the measurements showed a similar pattern for each of the 4 metabolites. Rises in levels during the first 25 days after anthesis were followed by a 15 day period during which the amounts per endosperm remained fairly constant. The determinations carried out on 45 day old samples, indicated that the amounts present of all metabolites fell towards the end of the sampling period. A comparison of the relative values of ADP and UDP throughout endosperm development showed that the levels of ADP were slightly higher except at 30 days after anthesis. The recovery data (table 3.1) indicate that the results for ADP were an underestimate, both with respect to the standard and to UDP. ADP-glucose and UDP-glucose had essentially the same recovery rates (table 3.1). The determination of these 2 metabolites per endosperm showed consistently higher levels for UDP-glucose. The ratios of UDP-glucose to ADP-glucose varied from as high as 4 at 12 days after anthesis to an average of 2 between 25 and 40 days after anthesis.

Amounts of ATP per endosperm were only determined at 25 and 45 days after anthesis, while UTP was measured only at the younger age. Estimates of the amounts of ATP and UTP at this younger age were approximately 3 and 7 n moles per endosperm respectively, while the amount of ATP was found to be 2 n moles per endosperm at 45 days after anthesis.

When the method of perchloric acid extraction was initially investigated, a comparison was made with an alternative hot ethanol procedure (section 3.2.3.3). However this latter procedure proved to be less efficient in terms of both protein precipitation and metabolite extraction. Only 63 per cent of the total protein in an extract prepared from endosperms (30 days after anthesis) was precipitated by hot ethanol while perchloric acid extraction removed 83 per cent of the protein from a duplicate sample.

Additionally, perchloric acid extraction gave substantially higher amounts of sugars and hexose phosphates. These hexose phosphates could not be detected in the hot ethanol extracts.

Nucleoside diphosphate sugars and nucleoside di- and triphosphates were not analysed in the hot ethanol extracts. However the stability of these metabolites to extraction by increasing concentrations of perchloric acid was investigated (section 3.2.3.4). HPLC analysis of these metabolites showed that at 3M perchloric acid, substantial breakdown of ADP- and UDP- glucose occurred, while at 1M perchloric acid all the metabolites, as determined by HPLC, were stable. Accordingly 1M perchloric acid was adopted for routine extractions.

### 3.4 Discussion

#### 3.4.1 Sampling of plant material and presentation of data

The results discussed previously in section 2.4 were obtained from relatively large samplings of plant material from a field grown crop. However the data which will be discussed in this and future sections have been determined from smaller samplings deriving from glasshouse grown plants. The samplings for metabolite determinations were carried out in quadruplicate and it was decided to present the results of each metabolite as individual determinations. This was a more valid procedure than transposing the data to mean values and applying various statistical operations which have a number of underlying assumptions, including that of the normal distribution. A common feature, in the presentation of biological data, is the application of standard error of the mean (SEM) to results which derive, often, from triplicate samplings. SEM imparts a calculated range of error to the data which may obscure the true range of the determinations. During the course of this present work Chevalier and Lingle (1983) used SEM to present results for the amounts of sucrose, glucose and fructose in

developing wheat endosperms. Their data for sucrose show a calculated range of error which implied levels of sucrose per endosperm that were approximately 4 times higher than those reported in the present work. These apparently high levels were misleading, since the width of the calculated error ranges suggest substantial variation in the determinations of sucrose used to compute these ranges. Additionally, since triplicate samplings were taken at each developmental age, no more than 3 results would accrue at each stage of the time course. Clearly there can be no possible advantage in applying SEM to these data, since a further set of 3 points arises i.e. the mean and 2 others represented by the extremes of the calculated range of error.

One of the major objectives of statistical analysis is to present large amounts of data in a concise, summarised form. Where large amounts of data have not been generated, it is more meaningful to show all of the determinations by means of a scatter diagram (M. Prentice, personal communication). Linear trends in the data can be analysed by a linear regression line, using the method of least squares (section 2.2.5).

#### 3.4.2 Metabolite determinations

In this work sucrose levels in the endosperm did not change appreciably during development. These levels were approximately 14 times higher than fructose and 40 times higher than glucose throughout the period of rapid dry weight accumulation (23-40 days after anthesis). However the values for fructose may have been underestimated by as much as 40 per cent. When this is taken into account, together with a probable overestimate for sucrose, then the differential in favour of sucrose may be approximately 7 times i.e. 7 times more sucrose than fructose.

Kumar and Singh (1984) showed a quite different pattern for sucrose levels in developing wheat grains, estimating that these levels

reached a maximum early on in development, thereafter declining to constant amounts. However their data was obtained from ethanolic extracts of whole grains. The results obtained during the course of the present work suggest that ethanol extraction was not an efficient means of metabolite extraction from wheat endosperms. Additionally, results pertaining to whole grains, which contain pericarp/testa, embryonic and endosperm tissue, are not applicable to the endosperm itself. Chevalier and Lingle (1983), while using endosperm extracts of developing wheat caryopses, showed levels of fructose and glucose that were extremely variable and in many instances were unable to detect quantifiable levels of either sugar. These variable results may have reflected the chemical methods of analyses which were used to determine their data.

The results for glucose and fructose, as determined in the current work, showed quite different patterns during endosperm development. These differences initially suggested that hydrolysis of sucrose by invertase was not the preferred pathway of sucrose catabolism. However the estimates of the 2 sugars during the early stages of endosperm development (12-20 days after anthesis) were not so different as to discount the involvement of invertase in these early stages.

The linear drop in glucose levels per endosperm during development may have been the result of rapid metabolism of that sugar, presumably in the first instance to G6P. Certainly this hexose phosphate was present in consistently higher amounts than either G1P or F6P during endosperm development. Alternatively, the drop in glucose levels per endosperm might have been due to a lack of production of this sugar rather than rapid catabolism. A role for sucrose synthase in the production of UDP- glucose and fructose from sucrose and UDP would be consistent with this hypothesis. However the UDP- dependent sucrose synthase reaction predicts equal amounts of UDP- glucose and fructose. The results obtained during the course of the present work suggest that this was not the case.



Notwithstanding this general observation, it was evident that the difference between the two metabolites was lowest during the period of rapid dry weight accumulation.

The situation might be made more tenable by the participation of UDP-glucose pyrophosphorylase which could facilitate the P<sub>Pi</sub>-dependent production of GlP and UTP from UDP-glucose. Turner and Turner (1975) have suggested such a role for this enzyme. Catabolism of UDP-glucose would bring about the generation of GlP which might then be available as a substrate for the ADP-glucose pyrophosphorylase reaction.

Fructose was apparently metabolised at a relatively slower rate than either glucose or UDP-glucose. However since fructose was present at levels approaching 14 times less than the amounts determined for sucrose it is conceivable that substantial metabolism of fructose might occur. If this was not the case then the amounts of fructose per endosperm might eventually accumulate to such an extent that the efficiency of either the sucrose synthase or possibly the invertase reaction might be inhibited. Fructose is known to inhibit the UDP- dependent sucrose synthase reaction (Avigad, 1982).

The determinations for ADP, UDP, ADP-glucose and UDP-glucose respectively per endosperm all showed rises in levels immediately prior to the period of rapid dry weight accumulation. This suggest that the regulation of their biosynthesis might be an important factor in establishing the onset of this period.

ADP-glucose and UDP-glucose both remained fairly constant between 25 and 40 days after anthesis and this could be consistent with their utilisation as metabolic intermediates in the conversion of sucrose to starch. Since the results do not give any indication of how these nucleoside diphosphate sugars might be distributed within the endosperm it would not be incorrect to suggest that both may be

substrates for starch synthesis. However current research indicates that the activity of ADP-glucose pyrophosphorylase is mainly amyloplastidic (Preiss *et al.*, 1985), although this evidence was derived from suspension cultures of soybean (MacDonald and Ap Rees, 1983) and may not be applicable to wheat endosperm cells.

During the period of rapid dry weight accumulation the levels of UDP-glucose were approximately twice those of ADP-glucose. These observations were in agreement with earlier work on wheat endosperms (Jenner, 1968), although in this instance very limited data was available, since the nucleoside diphosphate sugars were determined only at two developmental stages. Kumar and Singh (1984) suggest that the ratio of UDP-glucose to ADP-glucose was nearer to 3. However, as has been pointed out previously, their data is scarcely valid since it derives from whole grain extracts.

Interestingly, although the levels of UDP-glucose were higher than ADP-glucose, the amounts of ADP per endosperm were present in slightly higher levels than UDP. This would seem to suggest that ADP- dependent sucrose synthase activity was not favoured in wheat endosperms.

The data for UDP levels per endosperm indicate possible peak amounts around 30 days after anthesis and it may be that the availability of UDP during endosperm development is rate limiting with respect to the conversion of sucrose to UDP- glucose. This has implications for the activity characteristics of sucrose synthase and these will be discussed in a future section. An alternative pathway for the synthesis of UDP-glucose exists via UTP- dependent UDP-glucose pyrophosphorylase and this might explain the higher levels of UDP-glucose per endosperm in spite of the potential rate limiting amounts of UDP. However this would not be consistent with the proposed catatabolic role for PPi- dependent UDP-glucose pyrophosphorylase as discussed previously (section 3.4.2).

Measurements of the in vivo metabolite concentrations have not generally been reported in this present work since it was not feasible to assign estimations of endosperm water content to cellular compartments. However minimum calculations of endosperm sucrose concentration (data not shown) suggest a level of approximately 60mM during the period of rapid dry weight accumulation. This value was similar to earlier estimations for whole endosperm sucrose concentration (Jenner, 1974), although in that instance the result was derived from measurements at one developmental age only.

Minimum concentration range estimations for UDP and G1P, between 23 and 40 days after anthesis, were 0.08-0.24mM and 0.65-1.30mM respectively and these values may be significant with respect to the efficiencies of sucrose synthase and ADP-glucose pyrophosphorylase activities in developing endosperms.

### 3.5 Conclusions

Although sucrose levels per endosperm showed little variation throughout development this does not imply that dry weight accumulation was not limited by levels of endosperm sucrose. The results give no indication of how sucrose, or indeed the other metabolites, may have been partitioned between the apoplast and the symplast of the endosperm. Clearly, sucrose has to be available to the enzyme systems within the endosperm and control of this availability may be an important factor in the regulation of starch biosynthesis.

The quite different levels of glucose and fructose, that were observed during the middle and late stages of endosperm development, seemed to suggest an apparent lack of invertase activity or differential rates in the metabolic conversion of these hexose sugars. Alternatively, significant sucrose synthase activity might account for the higher levels of fructose. Invertase activity

may be more important during the early stages of endosperm development and this may be the reason why the amounts of glucose and fructose determined during these stages were quite similar. Unfortunately it was not possible to recover intact endosperms younger than 12 days after anthesis (section 2.3.3) and it is conceivable that invertase may play an important role in the immediate days post-anthesis, when endosperm cellularisation and cell division takes place (Duffus and Cochrane, 1982).

Although curve fitting techniques were not employed in the preparation of the results presented in this thesis, the data for UDP levels per endosperm may have implied a rise and fall of that metabolite during endosperm development. Since UDP would be utilised in the cleavage of sucrose by sucrose synthase to form UDP-glucose and fructose, the levels of UDP might be crucial in determining how efficiently sucrose is metabolised by endosperm cells. The levels of ADP-glucose per endosperm were lower than UDP-glucose per endosperm in spite of elevated amounts of ADP with respect to UDP and this might mean that ADP was not an efficient substitute for UDP in the sucrose-dependent sucrose synthase reaction. If this were the case then the formation of ADP-glucose would require to be catalysed by ADP-glucose pyrophosphorylase which has been consistently implicated in the regulation of starch biosynthesis (Preiss *et al.*, 1985), although most of the evidence has been derived from leaf and bacterial ADP-glucose pyrophosphorylases.

## CHAPTER FOUR

### 4.0 Sucrose synthase and invertase in developing wheat endosperms

#### 4.1 Introduction

Previously (section 3.4.2), the determined metabolite levels in developing wheat endosperms were discussed in relation to enzyme systems that may be involved in the conversion of sucrose to starch. The rate of dry matter accumulation, principally starch, might be related to the efficiency of these enzyme systems (section 3.1).

Sucrose synthase catalyses the reversible conversion of UDP and sucrose to UDP-glucose and fructose. This reaction has been implicated as the first step in the conversion of sucrose to starch (Turner and Turner, 1975; Nelson, 1980). In maize endosperms from mutant cultivars, reduced levels of starch have been linked to deficiencies in sucrose synthase activity (section 1.5) although other enzyme activities may also have been low. Presently this phenomenon has not been demonstrated in wheat.

The direct hydrolysis of sucrose by invertase is an alternative mechanism for the catabolism of sucrose. However the hydrolytic action of invertase is considered to be energetically wasteful, since subsequent metabolism of glucose and fructose may involve hexokinase activity with concomitant hydrolysis of ATP.

The data determined in section 3.3 suggest that invertase activity, at least during the middle and late stages of endosperm development, might not be the major activity. This conclusion was derived from comparing levels of glucose to fructose during endosperm development, although it was pointed out (section 3.5) that varying rates of hexose metabolism may also account for these differences. UDP and UDP-glucose levels rose prior to the onset of

rapid dry weight accumulation and this would be consistent with a developmental role for sucrose synthase. However low levels of UDP might serve to regulate the activity of sucrose synthase by rate limiting the conversion of sucrose to UDP-glucose and fructose.

Published data relating to sucrose synthase and invertase activities in wheat is extremely limited. Meredith and Jenkins (1976) have studied these enzymes in wheat caryopses. Unfortunately, since the extracts contained pericarp, embryo and endosperm tissue, it was not possible to distinguish the origin of the enzyme activity measured. Additionally they assayed sucrose synthase activity in the direction of sucrose synthesis. Clearly it is hardly valid to assign such data to sucrose/starch metabolism in endosperm tissue.

The current work describes the estimated levels of sucrose synthase and invertase activities in developing wheat endosperms and will be discussed in relation to the levels of metabolites previously determined in section 3.3.1. The amounts of UDP and sucrose consistent with maximum initial reaction velocities of sucrose synthase (cleavage reaction) are reported in consideration of estimated rates, in vivo, of endosperm dry weight accumulation.

## 4.2 Methods

### 4.2.1 Plant material

Plant material was grown and sampled essentially as previously described in section 3.2.1.

### 4.2.2. Reagents

2- [ N- morpholino ] ethane sulphonic acid (MES) and 3- [ N- morpholino ] propane sulphonic acid (MOPS) were obtained from Sigma Chemical Co. Ltd., Poole, Dorset.

All other reagents were of analytical grade or of the highest purity available.

#### 4.2.3 Time course study of sucrose synthase and invertase activities

Samplings for the time course study of sucrose synthase and invertase activities were obtained from a set of 18 pots containing 6 plants each. At various stages during endosperm development, 18 ears were sampled as described in section 3.2.1. The cut ears were separated into 3 lots of 6 and caryopses removed as previously described (section 3.2.1). Endosperms were aged according to the system described in section 2.4.3 and numbers (15-30 depending upon stage of development) were prepared for enzyme analysis as described in the next section.

#### 4.2.4 Preparation of enzyme extracts for enzyme studies

Endosperms (15-30 depending upon the stage of development) were dissected free of pericarp/testa and embryonic tissues and placed in 2-3ml of ice cold MOPS buffer (50mM) at pH 7.0. The endosperms were then homogenised on ice for 5 min using a hand-held Potter-type all-glass homogeniser. The resulting homogenate was centrifuged for 15 min at 2°C and 10 000g ( $r_{av} = 11.2\text{cm}$ ) in an MSE High Speed 18 centrifuge. The final supernatant was utilised immediately for the determination of enzyme activities.

#### 4.2.5 Determination of sucrose synthase activity

Sucrose synthase (EC 2.4.1.13) activity was determined essentially according to the method of Avigad and Milner (1966). The enzyme digest, unless otherwise stated, was composed of (mM): MOPS, pH 7.0, 50; sucrose, 250; UDP, 5; NaF, 5 and distilled water to 0.9ml. The digest was pre-incubated in a water bath at 25°C for 10 min and the enzyme reaction initiated by the addition of an aliquot (0.1ml) of the supernatant derived from the procedures described in

sections 4.2.3/4. At suitable intervals, e.g. 5 min, aliquots (0.18ml) were removed from the digest and added, with mixing, to test-tubes containing 1mM N-ethylmaleimide (0.82ml) which was then placed in a boiling water bath for 1 min. Following removal of the tubes and cooling to room temperature, the contents of the tubes were assayed for reducing sugars by the Somoygi-Nelson procedure (Nelson, 1944). Somoygi-Nelson copper reagent (1ml) was added to each tube and, following thorough mixing, the tubes were capped and placed in a boiling water bath for 10 min. After heating, the tubes were removed from the bath and allowed to cool before adding the Somoygi-Nelson arsenomolybdate reagent (1ml). The contents of the tubes were well mixed and made up to 10ml with distilled water. After 15 min, but not more than 40 min, the absorbances were read at 500 nm in a Pye Unicam SP 8/100 UV/Vis spectrophotometer. A standard calibration curve of an equi-molar glucose/fructose mixture with a range of concentrations from 0-3.0  $\mu$  moles hexose per ml was prepared with each set of enzyme assays. For each activity determination controls in the absence of substrate(s) and in the absence of enzyme were carried out. The latter control was to check for non-enzymic hydrolysis of sucrose while the former was utilised to give a simultaneous determination of invertase activity as described in the next section.

#### 4.2.6 Determination of invertase activity

Invertase (EC 3.2.1.26) activity at pH 7.0 was determined as described for sucrose synthase activity (section 4.2.5) except that UDP was omitted. Invertase activity at pH 5.0 was assayed in a similar manner except that the digest buffer was composed of sodium acetate (50mM) at pH 5.0. Control assays were as previously described (section 4.2.5).

#### 4.2.7 Linearity of response

The method of analysis for sucrose synthase and invertase activity (sections 4.2.5/6) allowed for continual monitoring of the



linearity of response and there was no requirement for separate control experiments.

#### 4.2.8 Determination of sucrose synthase and invertase activity in pericarp/testa tissue

Pericarp/tissue was prepared in exactly the same manner as described for endosperms (section 4.2.4) except that endosperm and embryonic tissues were discarded. Determinations of sucrose synthase and invertase activities were carried out as described for their analagous endosperm activites in sections 4.2.5/6/7.

#### 4.2.9 Analysis of soluble protein

Endosperm extracts, derived from the procedures described in section 4.2.4, were dialysed overnight in a refrigerator at 4°C. The dialysing medium was distilled water and the ratio of dialysate to dialysing medium was 0.01. A magnetic stirrer was used to circulate the dialysis medium. Following dialysis the dialysate was suitably diluted and aliquots (1ml) were analysed for soluble protein as described in section 3.2.3.5.

#### 4.2.10 pH-dependent endosperm sucrose synthase activity

The pH-dependent activity of endosperm sucrose synthase was investigate using an extract of endosperms (30) with a morphological age of 33 days after anthesis prepared as above (section 4.2.4) except that approximately 4.5ml of homogenisation buffer was used. Sucrose synthase activity was determined in aliquots (0.1ml) as a function of varying pH. The following buffers were used as substitutes for the buffer described in section 4.2.5 (pH): glycyl glycine/HCl, 3.0/3.5; sodium acetate, 4.0/4.5/5.0; MES, 5.5/6.0/6.5; MOPS, 7.0/7.5; tricine, 8.0/8.5 and glycine/NaOH, 9.0/9.5/10.0. Assays and controls were as described in section 4.2.5 and all buffer concentrations were 50mM.

#### 4.2.11 pH- dependent pericarp/testa invertase activity

The pH- dependent invertase activity from pericarp/testa tissue was determined in material derived from caryopses (40) with a morphological age of 25 days after anthesis. Tissue preparation was as described in section 4.2.8 and the method of assay was as previously detailed for endosperms in section 4.2.6. The range of buffers was the same as for the determination of pH-dependent sucrose synthase activity in endosperms (section 4.2.10).

#### 4.2.12 Enzyme kinetics

Values for the concentrations of the substrates (UDP and sucrose) required to give initial reaction rates at 50 per cent of the apparent maximum velocity were determined for endosperm sucrose synthase. These parameters were measured using endosperm extracts from endosperms with a morphological age of 30 days after anthesis. The extracts were prepared as described in section 4.2.10 and the method of assay was essentially as previously detailed in section 4.2.5 except for the following modifications: UDP-dependent initial reaction velocities were determined by varying the concentration of UDP in the assay digest from 0-20mM, keeping all other components constant. Sucrose-dependent initial reaction velocities were carried out in a similar manner except that the concentration of sucrose was varied from 0-750mM. All other digest components were kept constant. Sampling was effected every 80s up to 320s. The data for UDP- and sucrose-dependent initial reaction velocities were plotted with initial velocities ( $v_o$ ) as a function of substrate concentration ( $s$ ) and the apparent Michaelis constant ( $K_m$ ) determined from replotting the data according to the following equations:

$$\frac{1}{v_o} = \frac{K_m}{V} \frac{1}{s} + \frac{1}{V} \quad (1)$$

$$\frac{s}{v_o} = \frac{1}{V} s + \frac{K_m}{V} \quad (2)$$

where  $v_o$  = initial velocity,  $V$  = maximum velocity,  $s$  = substrate concentration and  $K_m = s$  when  $v_o = \frac{V}{2}$

#### 4.2.13 Inhibition studies

The possible presence of endogenous endosperm inhibitors of endosperm sucrose synthase was investigated by determining the activity of sucrose synthase in individual endosperm extracts from endosperms of differing morphological ages (15, 33, 40 and 44 days after anthesis respectively) and in combined extracts (15/33, 40/33 and 44/33 days after anthesis respectively). Each extract was prepared as described in section 4.2.4, from the following numbers of endosperms (no.): 15, 25; 33, 25; 40, 15 and 44, 15. The activities were determined as described in section 4.2.5. For the combined extract activities, aliquots (0.1ml) of each extract made a total of 0.2ml for initiation of the enzyme reaction. Duplicate determinations were carried out and the controls were as previously described (section 4.2.5).

Pericarp/testa invertase activity (at pH 5.0) was investigated in a similar manner except that caryopses (44) with a morphological age of 23 days after anthesis were used as the source of pericarp/testa and endosperm extracts respectively. The pericarp/testa extract was prepared from all of the caryopses (44) as described in section 4.2.8, while the endosperms were split into 3 lots of 11 prior to extraction as described in section 4.2.4. Invertase activity was assayed at pH 5.0 in each extract as detailed in sections 4.2.6/8 and then re-assayed in the pericarp/testa extract but in the presence of an aliquot (0.1ml) of each of the endosperm extracts respectively. The complete experiment was then repeated using a fresh extract of pericarp/testa tissue as described above, but using endosperm extracts derived from endosperms with a morphological age of 33 days after anthesis.

Figure 4.1 The activities of sucrose synthase and invertase during endosperm development

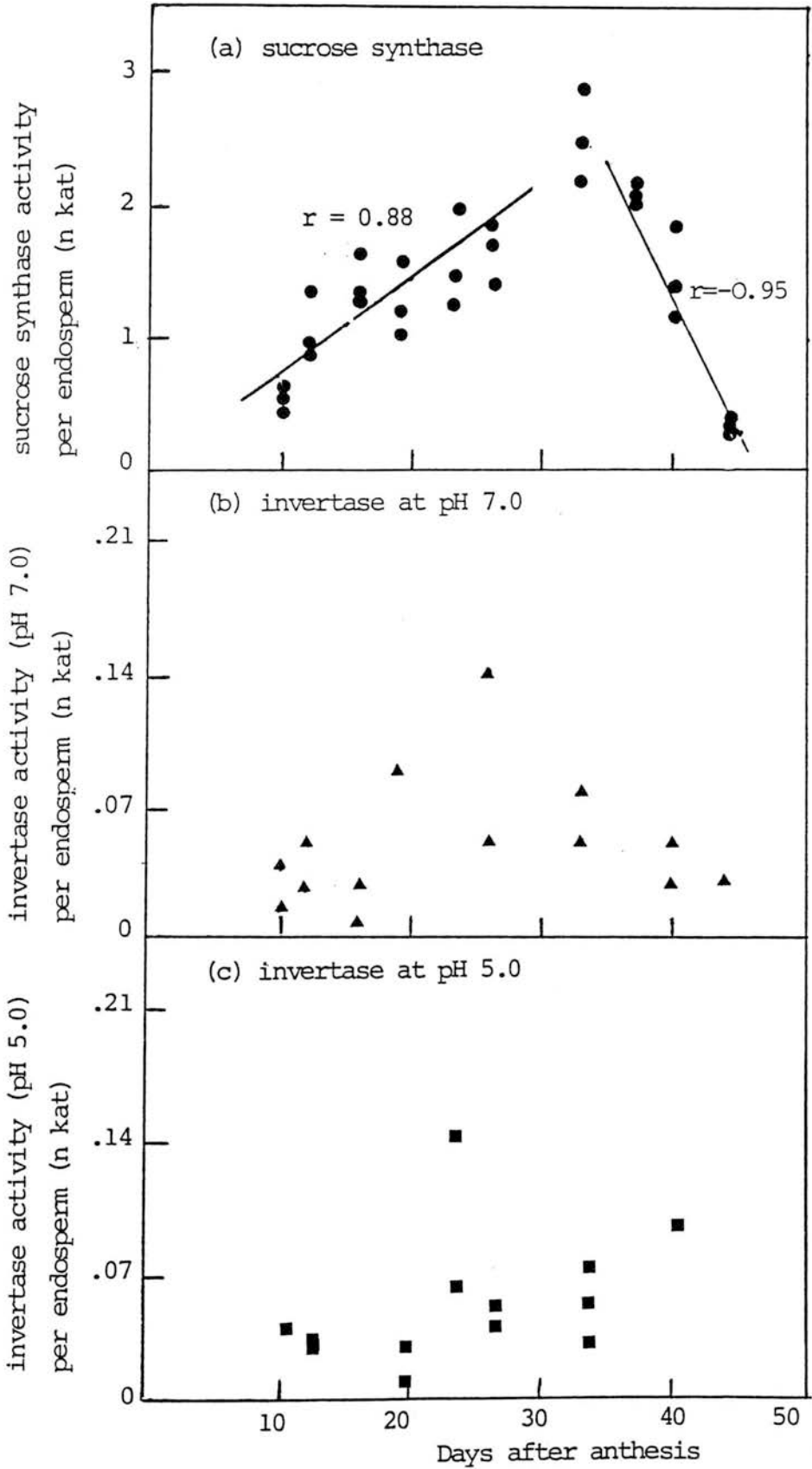
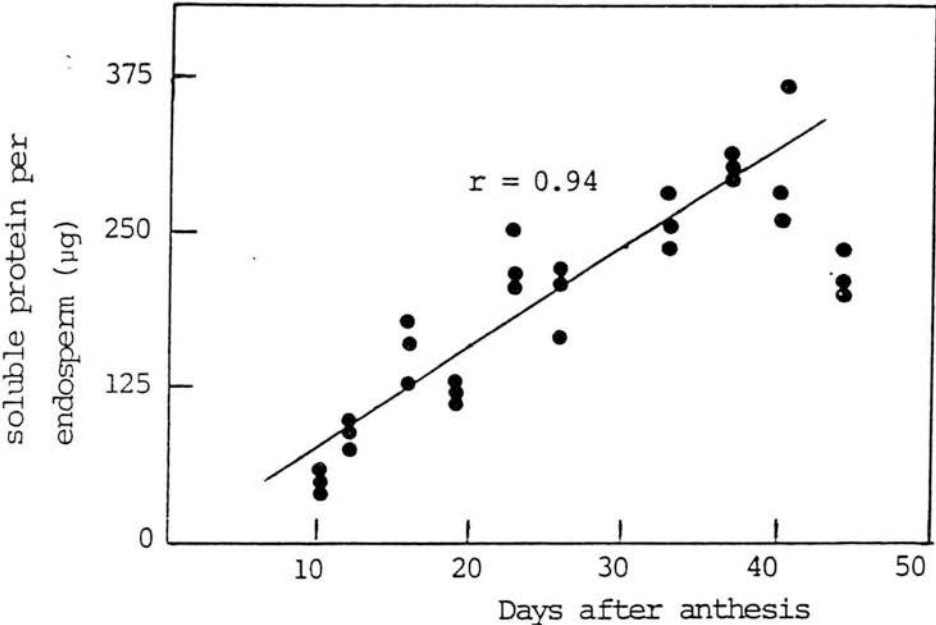


Figure 4.2 Determination of soluble protein in developing endosperms 75.



#### 4.2.14 Presentation of data

Where appropriate, the data have been presented as described previously in section 3.4.1 and analysed according to the procedures outlined in section 2.2.5.

### 4.3 Results

#### 4.3.1 Sucrose synthase and invertase activities during endosperm development

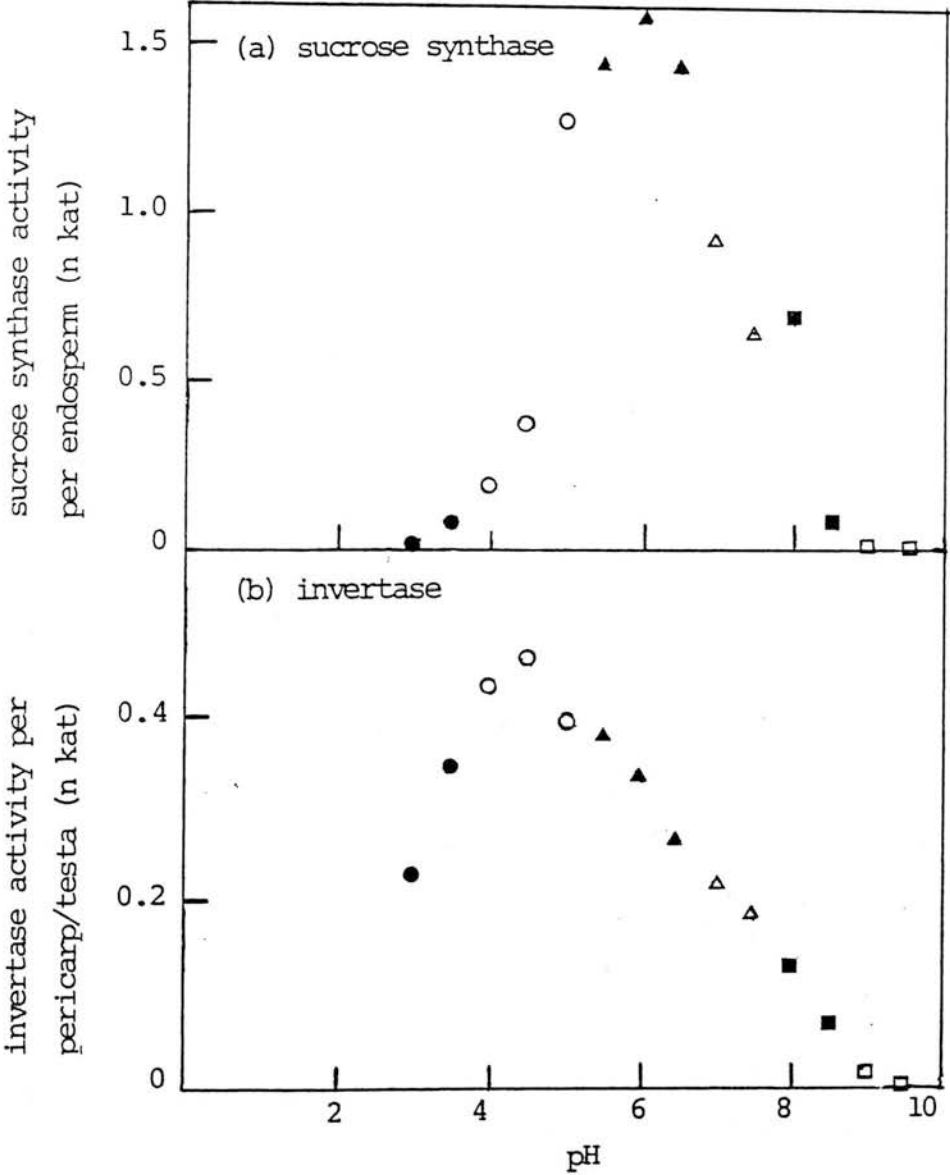
The activities of sucrose synthase and invertase (pH 7.0 and pH 5.0) in developing endosperm extracts are shown in fig 4.1. The determined activities have been expressed in n kat per endosperm.

Sucrose synthase activity (fig 4.1a) rose steadily during the first 33 days of endosperm development but fell sharply during the later stages of development. The rise in activity was characterised by a linear regression line ( $r = 0.88$ ), with 78 per cent of the data having a linear relationship with developmental time (days after anthesis). The sharp decline had a high negative correlation ( $r = -0.95$ ) and the measured levels of activity at 44 days after anthesis were approximately 12 per cent of those determined at 33 days after anthesis.

Invertase activity at pH 7.0 (fig 4.1b) and at pH 5.0 (fig 4.1c) was detected in about 50 per cent of all samples assayed. The levels at both orders of pH were approximately 30 times less than those obtained for sucrose synthase. The data were scattered and exhibited no apparent trend.

Fig 4.2 shows the results from the determinations of soluble protein in the extracts analysed for enzyme activities. Measurements during the period up to 40 days after anthesis show a linear increase in soluble protein with a high positive correlation ( $r = 0.94$ ). A slight drop was recorded for the samples assayed at

Figure 4.3 pH- dependent activities of endosperm sucrose synthase<sup>77</sup> and pericarp/testa invertase



Key: glycyl glycine/HCl (●), sodium acetate (○), MES (▲), MOPS (△), tricine (■) and glycine/NaOH (□)

44 days after anthesis.

During the developmental period, precipitates deriving from the centrifugation procedures (section 4.2.4) were analysed occasionally for bound enzyme activity (sucrose synthase and invertase). No such activity was ever detected.

Samples of endosperms (10) at various stages of development were periodically assessed for bacteriological contamination by homogenisation in sterile 0.25 per cent v/v Ringers solution (2ml). These homogenates were then sent for bacteriological enumeration. Results consistently indicated less than 4 gram positive species and less than 16 gram negative species per 10 endosperms respectively. No yeasts were ever isolated. These results confirm insignificant bacterial contamination of the endosperms (Baxter, 1972).

#### 4.3.2 pH-dependent activity of endosperm sucrose synthase and pericarp/testa invertase

The pH-dependence of sucrose synthase activity was determined in endosperm extracts. A similar study for invertase was made difficult by the relatively low activity of that enzyme in the endosperm. Instead, a study of the pH-dependent activity of pericarp/testa invertase was carried out. The results for both enzymes are shown in fig 4.3. The data were expressed in n kat per endosperm and n kat per pericarp/testa.

The pH-dependent activity of endosperm sucrose synthase (fig 4.3a) followed a bell shaped curve with maximum activity determined in MES buffer at pH 6.0. The measured activity at pH 7.0 was approximately 60 per cent of that recorded at the optimum pH. Pericarp/testa invertase activity (fig 4.3b) exhibited a broader pH profile. The optimum pH was 4.5 and considerable activity was measured at pH 3.0 (about 50 per cent of the maximum). It was not possible to measure the activity at pH values less than 3.0 since



Figure 4.4 UDP- dependent endosperm sucrose synthase initial 79.  
reaction velocities ( 0 - 20 mM UDP )

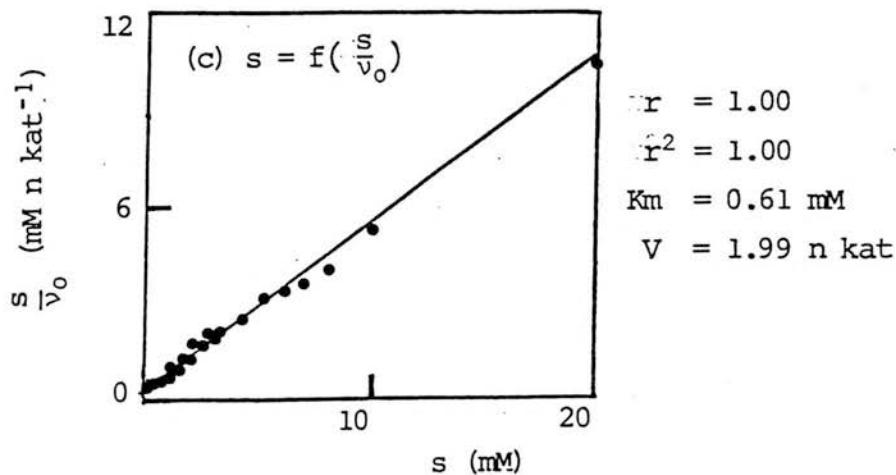
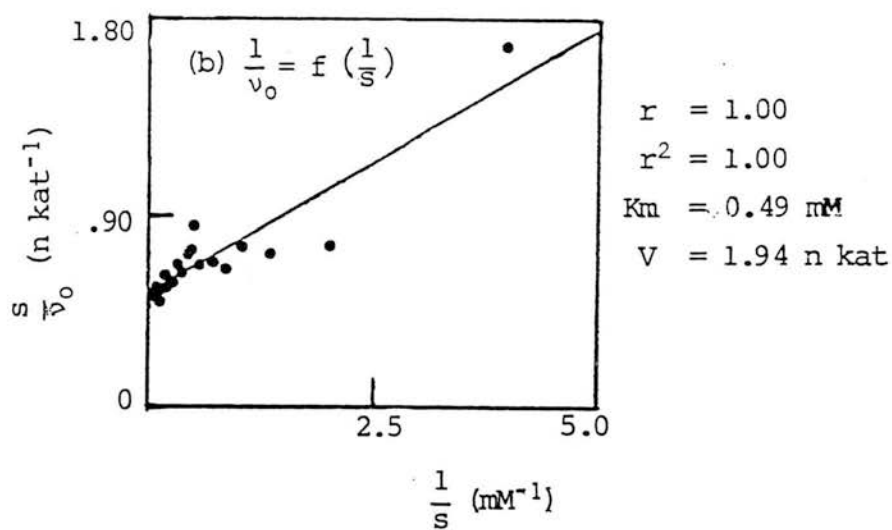
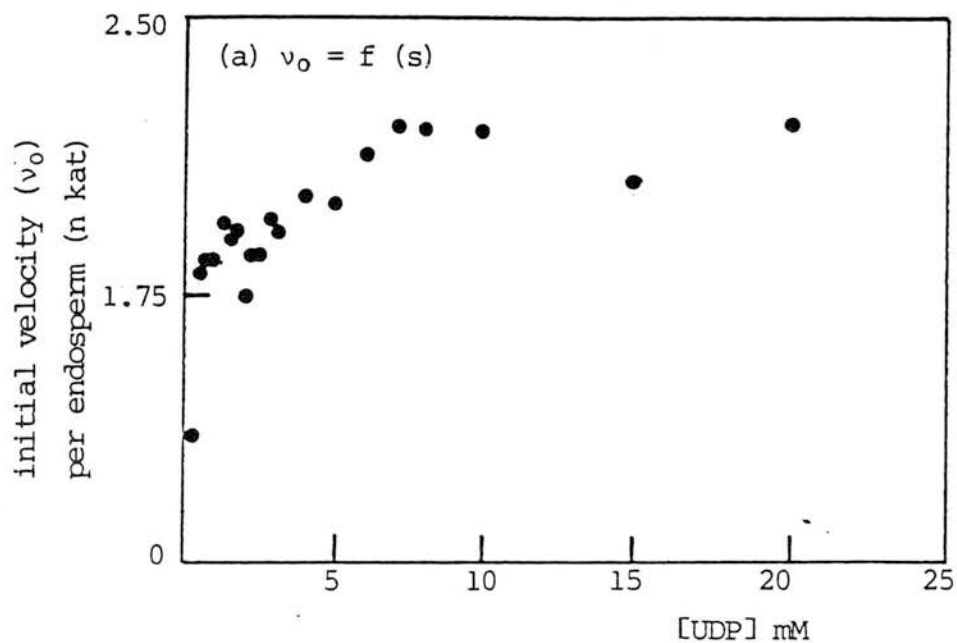


Figure 4.5 UDP- dependent endosperm sucrose synthase initial  
reaction velocities ( 0 - 10 mM UDP ) <sup>80.</sup>

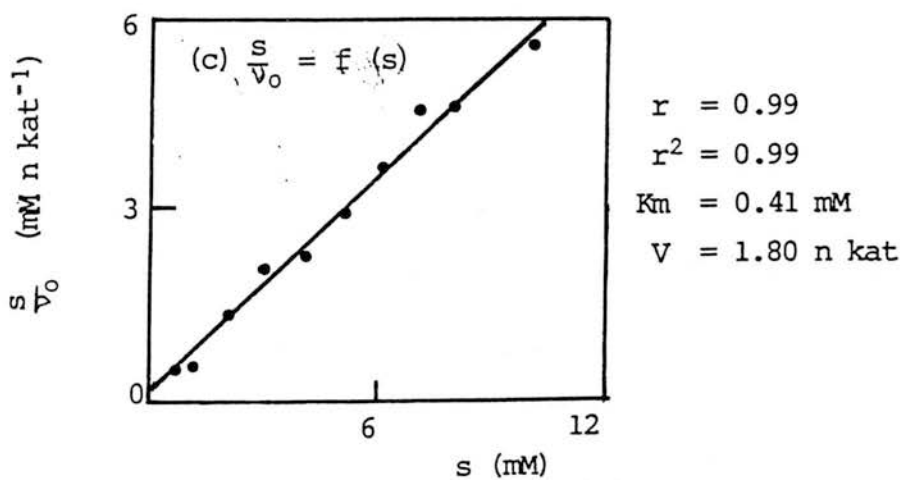
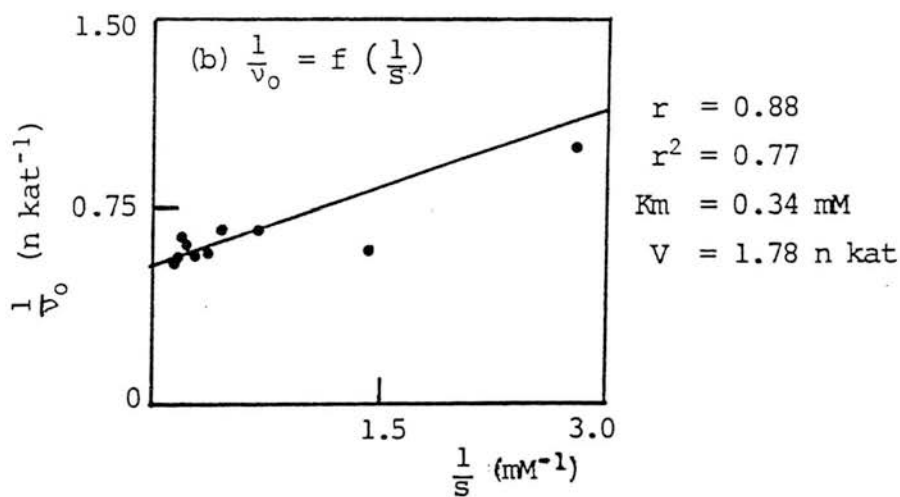
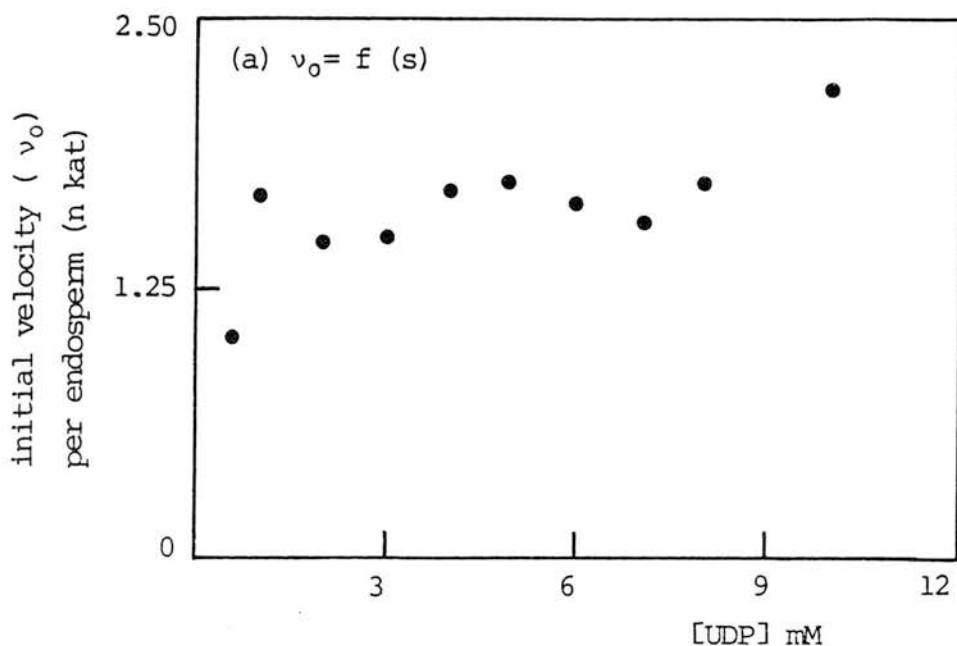
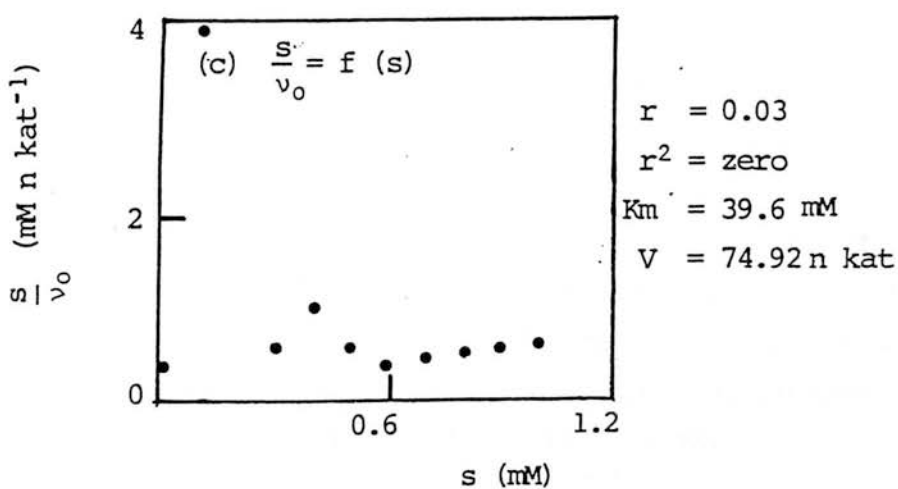
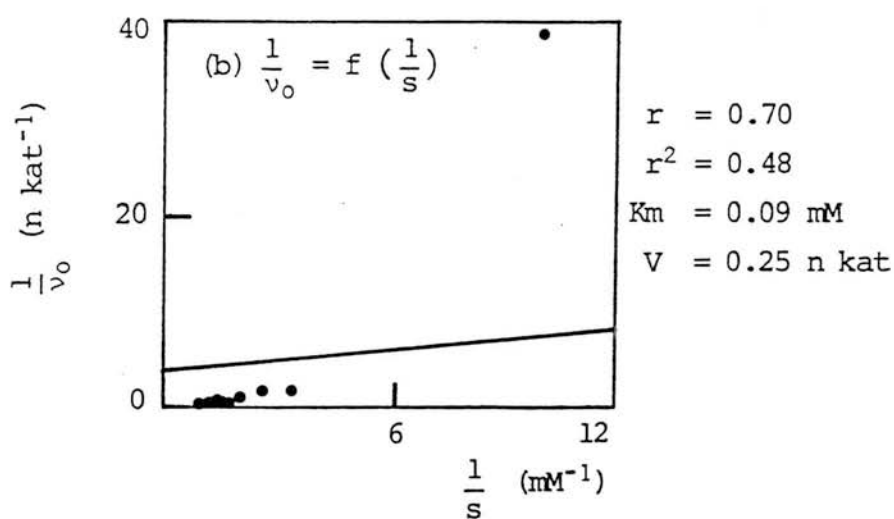
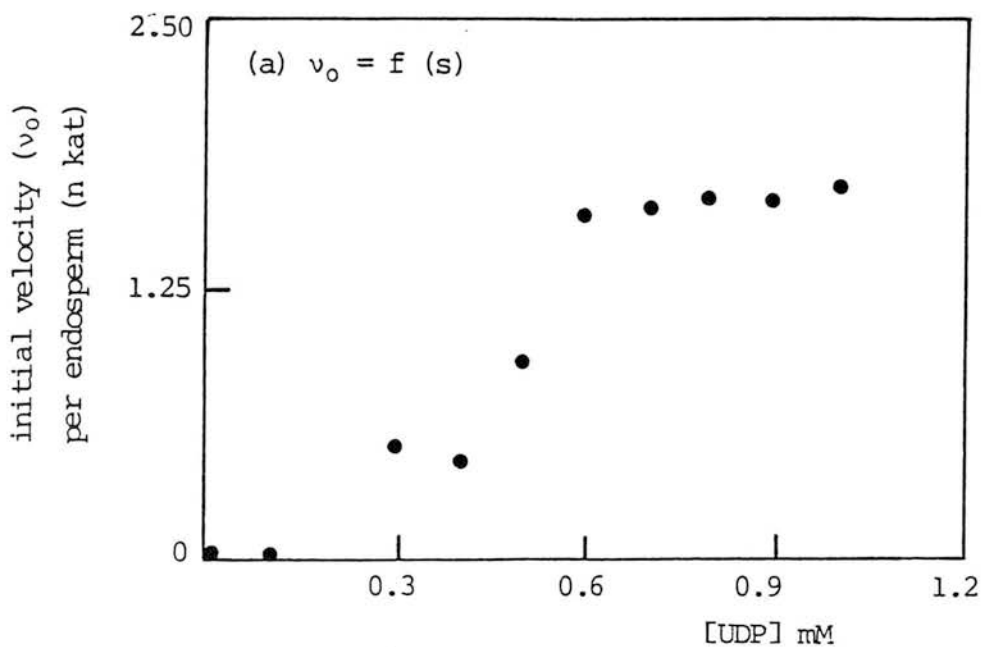


Figure 4.6 UDP- dependent endosperm sucrose synthase initial reaction velocities ( 0 - 1 mM UDP ) 81.



these conditions resulted in significant non-enzymic hydrolysis of sucrose.

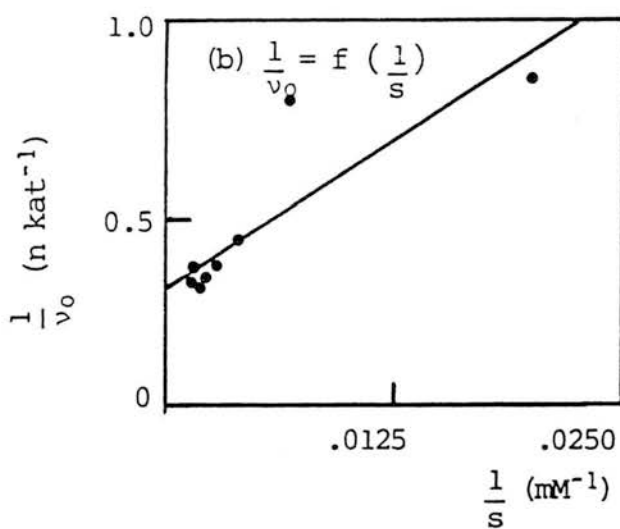
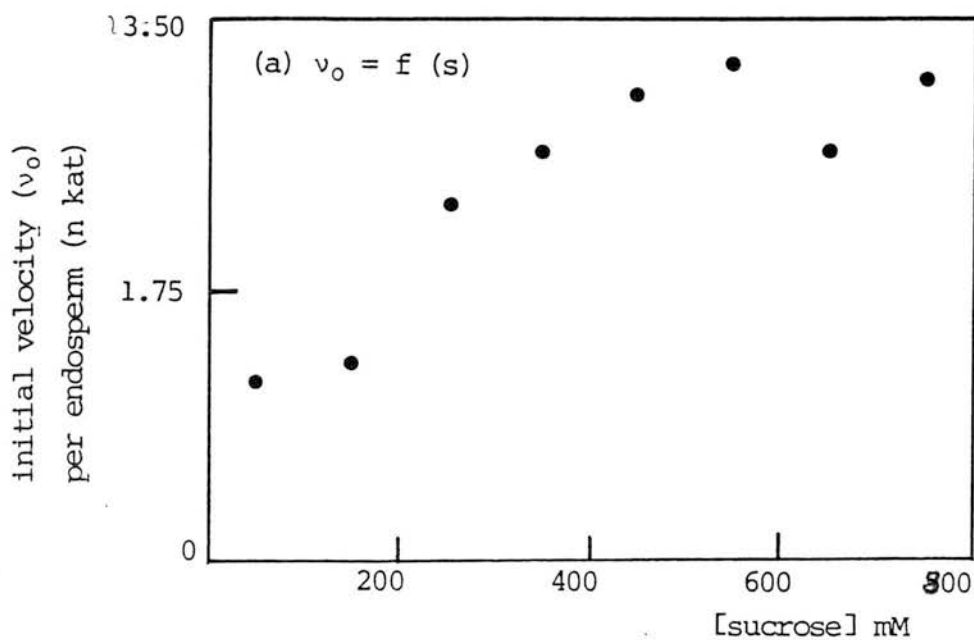
#### 4.3.3 Determinations of $K_m$ for UDP- and sucrose-dependent endosperm sucrose synthase initial reaction velocities

UDP-dependent sucrose synthase initial reaction velocities were determined over 3 concentration ranges while maintaining the concentration of sucrose constant, at 250mM.

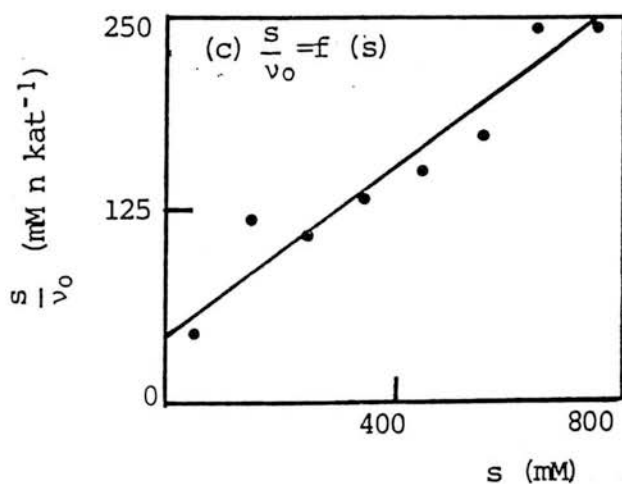
Fig 4.4 shows the results obtained when the concentration of UDP in the enzyme digest was varied from 0-20mM. In fig 4.4a the data were plotted as initial velocity ( $v_o$ ) versus substrate concentration ( $s$ ) while in figs 4.4b and 4.4c the data were replotted according to equations 1 and 2 respectively as detailed in section 4.2.12. These linear plots derive from the well-known Michaelis-Menten equation (Henderson, 1978) and are generally ascribed to Lineweaver and Burk and Hanes respectively (Dixon and Webb, 1979). The double reciprocal plot (fig 4.4b) gave a calculated  $K_m$  (UDP) of 0.49mM while the single reciprocal plot (fig 4.4c) shows a slightly higher  $K_m$  (UDP) of 0.61mM. The estimations of maximum velocity ( $V$ ) were similar when calculated by either procedure. In fig 4.5a the same parameters were determined but over a narrower concentration range. The  $K_m$  (UDP) when calculated by the double reciprocal method (fig 4.5b) gave a value of 0.34mM, however the degree of linearity ( $r = 0.82$ ) was poorer than that observed in fig 4.4b. When the data was replotted by the single reciprocal procedure (fig 4.5c) the results showed a high positive correlation ( $r = 0.99$ ) and gave a  $K_m$  (UDP) value of 0.41mM which was closer to the results obtained over the larger concentration range (fig 4.4). The results in figure 4.6a were obtained from varying the concentration of UDP in the enzyme digest from 0-1mM and show a kinetic pattern different from the predicted by Michaelis theory (Dixon and Webb, 1979). A lag phase occurred at substrate (UDP) concentrations less than 0.4mM and this phase was followed by a rapid increase in initial velocities between 0.4 and 0.6 mM UDP. Thereafter the rate of

Figure 4.7 Sucrose- dependent endosperm sucrose synthase  
initial reaction velocities ( 0 - 750 mM sucrose )

83.

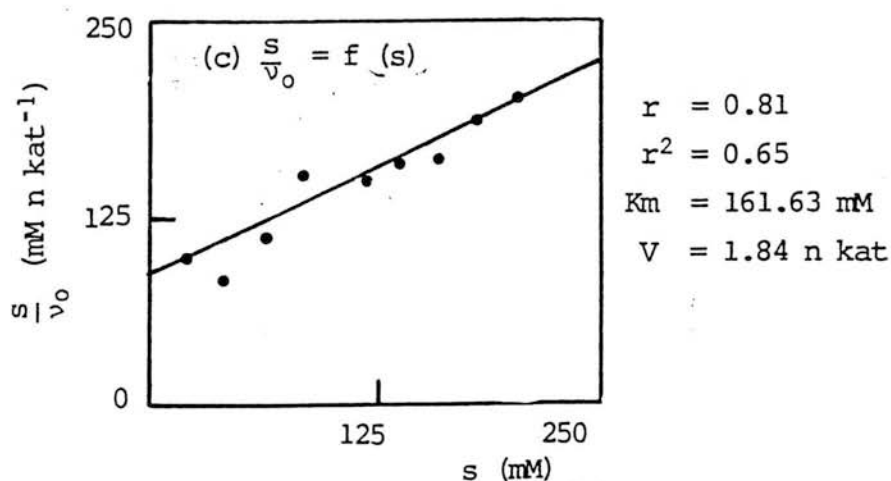
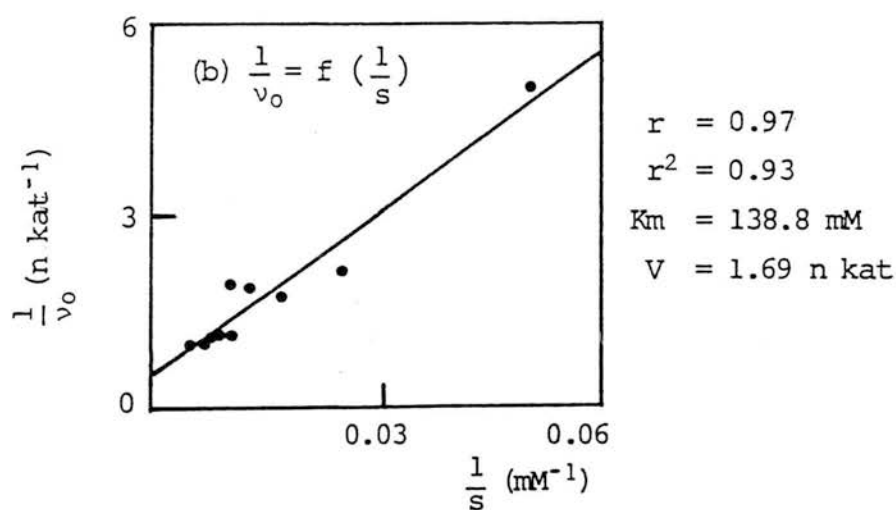
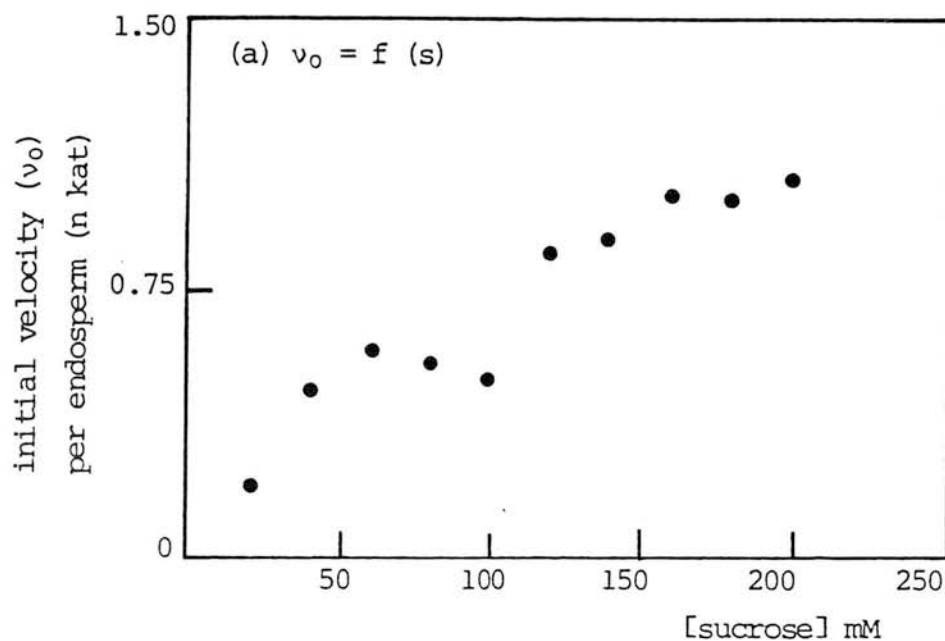


$r = 0.86$   
 $r^2 = 0.75$   
 $K_m = 91.8 \text{ mM}$   
 $V = 3.10 \text{ n kat}$



$r = 0.96$   
 $r^2 = 0.92$   
 $K_m = 169 \text{ mM}$   
 $V = 3.78 \text{ n kat}$

Figure 4.8 Sucrose- dependent endosperm sucrose synthase  
initial reaction velocities ( 0 - 200 mM sucrose )



product formation was approximately constant. Attempts to linearise the data (figs 4.6b and 4.6c) proved impossible and the respective calculated  $V$  values bore no relationship to the raw data (fig 4.6a).

Sucrose-dependent sucrose synthase initial reaction velocities were determined over 2 concentration ranges while maintaining the concentration of UDP constant, at 5mM.

Fig 4.7a shows the data obtained by varying sucrose in the enzyme digest from 0-750mM. The double reciprocal plot (fig 4.7b) gave a calculated  $K_m(\text{sucrose})$  of approximately 92mM, while the single reciprocal plot (fig 4.7c) shows an estimated  $K_m(\text{sucrose})$  of 169mM. This value was almost double that calculated by the plot in fig 4.7b.

The initial reaction velocities of sucrose-dependent sucrose synthase were re-determined over a narrower concentration range (0-200mM sucrose) and the results are shown in fig 4.8a. Both linear plots (figs 4.8b and 4.8c) gave calculated  $K_m(\text{sucrose})$  values significantly higher than those previously obtained from the data shown in fig 4.7b and were more in line with the higher determination obtained from the results as plotted in fig 4.7c.

#### 4.3.4 Endogenous inhibitor studies

The results from the combination experiments (section 4.2.13), designed to investigate the possibility of inhibition of endosperm sucrose synthase activity extracted from endosperms with relative high activity (33 days after anthesis), by extracts of endosperms from the early and late stages of development (15, 40 and 44 days after anthesis) are given in table 4.1.

Table 4.1 Combined extract sucrose synthase activity experiments

Endosperm age (days after anthesis)	Activity of extract (n kat digest <sup>-1</sup> )
15	0.36
33	0.74
15 + 33	1.02
33	1.11 (1.04)
40	0.10 (0.22)
33 + 40	1.25 (1.38)
33	1.66 (1.71)
44	0.13 (0.07)
33 + 44	1.81 (1.78)

Data in parenthesis represents duplicate analyses.

The data show no evidence of significant inhibition (or activation) when the 33 day old endosperm extracts were assayed in combination with either the young or the old endosperm extracts.

An analogous experiment (data not shown), but this time assaying pericarp/testa tissue for invertase activity in the presence of endosperm extracts, showed that the endosperm extracts did not inhibit invertase activity in the former tissue.

#### 4.4 Discussion

##### 4.4.1 Developmental studies

Throughout endosperm development, sucrose synthase had consistently higher activity than invertase. These levels, relative to invertase, ranged from 10 times higher during the early stages of endosperm development to as much as 50 times higher at 33 days after anthesis. During the course of this present work, Chevalier and Lingle (1983) reported levels of sucrose synthase activity, in developing wheat endosperms, that were approximately 25 per cent less than those obtained in the current investigation. However their data can only be of limited value since it was derived from



measuring the enzyme in the direction of sucrose synthesis. Since the synthesis of sucrose is contrary to the proposed in vivo role of sucrose synthase, measurement of its activity in this direction may not be a valid procedure (Crabtree et al., 1978). Dale and Housley (1986) have recently measured sucrose synthase activity in developing wheat endosperms and found general levels of activity similar to those reported in this present work. However they suggested that sucrose synthase activity did not vary significantly during endosperm development. This view is contrary to the observations reported in this thesis and was concluded from data derived only during the period of rapid grain filling. Dale and Housley (1986) did not measure sucrose synthase before or after that period. Additionally, their results originated from pre-frozen samples which were subjected to fairly lengthy procedures involving thawing, homogenisation, centrifugation and dialysis (8h) prior to assay of enzyme activity. These lengthy procedures may have given results that did not accurately reflect the activities in preparations from fresh tissues, particularly since the enzyme was assayed in the direction of sucrose synthesis.

The maximum rate of endosperm dry weight accumulation, reported in section 2.3.2, was 1.2mg per endosperm per day. This rate of dry weight accumulation was commensurate with a final grain dry weight of approximately 50mg and was in agreement with values obtained by other workers (Sofield et al., 1977a; Jenner, 1982). Endosperm dry weight accumulation of 1.2mg per day would require the incorporation of  $3.7 \mu$  moles of sucrose per day which relates to a catalytic rate of 0.04n kat. This calculation makes a number of assumptions. The first is that all of the dry weight accumulation was a function of starch biosynthesis. This is clearly not the case since reserve protein also increases during this period (Simmonds and O'Brien, 1981; Jenner, 1986) and eventually contributes between 10 and 15 per cent of the harvest-ripe endosperm dry weight (Kent, 1983b). The second assumption is that both the hexose moieties which make up the sucrose molecule are incorporated into starch. Lee and Su (1982) have fed double-labelled sucrose ( $^{14}\text{C}$ - glucosyl

-<sup>3</sup>H-fructoside) to detached ear cultures of rice and their results suggested a preference for the incorporation of the glucosyl component into starch. While this data cannot be directly extrapolated to the situation that may exist for wheat, a reasonable estimate for a required catalytic rate for the conversion of sucrose would be 0.09 n kat per endosperm i.e. approximately 7.5  $\mu$ -moles of sucrose per day. Thirdly, wheat endosperm starch biosynthesis may not take place evenly over a 24h period. Sakri and Shannon (1975) have shown that total wheat endosperm sugars did decline during the night, however this does not necessarily imply a reduced rate of starch biosynthesis since ample amounts of precursors, e.g. ADP-glucose, might be present. Finally, these calculations do not take into account the possibility of endosperm starch turnover. Watson (1985) has provided some evidence for this phenomenon in detached ear experiments with barley where there was an apparent loss of <sup>14</sup>C-label in endosperm starch relative to initial counts following <sup>14</sup>CO<sub>2</sub> pulse labelling.

The minimum levels of sucrose synthase activity between 20 and 40 days after anthesis were around 1 n kat per endosperm and this value was approximately 11 times the apparent catalytic requirement. However these determinations of sucrose synthase activity were made in vitro and in the presence of relatively high concentrations of sucrose (250mM) and UDP (5mM). Estimations of the concentrations of these metabolites in developing endosperms (section 3.4.2) showed levels that were about 4 times and 20 times less, respectively, than those used in the enzyme digest. The calculated Km values for sucrose and UDP suggests that, in both cases, the endosperm concentrations of these substrates may be insufficient to facilitate maximum enzyme activity (see section 4.4.2.2).

Maximum levels of sucrose synthase activity were found at 33 days after anthesis and these levels were apparently 30 times greater than the estimated catalytic requirement. These, and other, in

vitro measurements of sucrose synthase activity during endosperm development may have been underestimated since they were determined at pH 7.0, while the optimum pH was later shown to be around pH 6.0. Preiss (1982a) has stated that the optimum pH for sucrose synthase from cereals, tubers and legumes, ranges between pH 7.2 and pH 7.6. However this is a misleading statement, since it fails to define the direction of the reaction.

Sucrose synthase activity declined between 33 and 44 days after anthesis, while endosperm dry weight accumulation was observed to continue up to 40 days after anthesis. The decline in sucrose synthase activity between 33 and 40 days after anthesis was not apparently due to inefficient extraction of soluble protein, since the determinations of this parameter of endosperm development showed rising levels up to 40 days after anthesis. Since storage proteins are not soluble in simple aqueous buffer systems (Shewry and Mifflin, 1985), the values for soluble protein obtained during the course of this present work should not have been overestimated due to extraction of reserve protein. The continuation of endosperm dry weight accumulation, in spite of falling levels of sucrose synthase activity, may have been due to an increasing contribution by reserve protein. Since starch and reserve protein were not directly estimated during endosperm development it was not possible to assign respective proportions of these components to dry weight accumulation. However data obtained by Donovan *et al.*, (1976) do not show differing relative proportions of starch and protein during grain filling in wheat, although this data was derived from whole grain analysis and these conclusions may not apply to endosperms.

Invertase activity in developing endosperms was difficult to detect. However the measured levels of activity were not insignificant, since a catalytic rate as low as 0.01n kat per endosperm (lowest level of detectable invertase activity found in developing wheat endosperms) might account for the hydrolysis of approximately 0.9  $\mu$  moles of sucrose per day. Chevalier and Lingle

(1983) failed to detect any invertase activity in supernatant fractions from either wheat endosperm or wheat pericarp/testa preparations. During the course of the present work significant invertase activity was readily detected in extracts of pericarp/testa (approximately 0.3 n kat per pericarp/testa at 23 days after anthesis, results not shown) and it is possible that the relatively low and variable levels detected in the endosperm were due to contamination of endosperm extracts by pericarp/testa tissue. Interestingly, the pH profile of pericarp/testa invertase showed a maximum at pH 5.0. If the endosperm extracts had been contaminated with pericarp/testa invertase, it would not have been unreasonable to have observed significant differences, even in the variable levels of endosperm invertase activity that were detected, between assays at pH 7.0 and pH 5.0. Such differences were not apparent and it was concluded that endosperm invertase activity did not appear to be an artefact of the extraction procedure.

#### 4.4.2 UDP- and sucrose-dependent endosperm sucrose synthase initial reaction velocities

##### 4.4.2.1 Presentation of data

The apparent  $K_m$  values for the UDP- and sucrose-dependent endosperm initial reaction velocities were determined by 2 procedures. The first of these was the popular double reciprocal plot of reciprocal initial velocity versus reciprocal substrate concentration (Lineweaver and Burk, 1934). This commonly reported method of determining  $K_m$  has been shown to be the least reliable of all methods since it places too much reliance on the experimental data derived from the lowest levels of substrate concentration. At these levels, the greatest degree of experimental error may occur (Ferdinand, 1976; Dixon and Webb, 1979). It is possible to apply a weighted least-squares fit to the double reciprocal plot (Henderson, 1978), however this procedure requires a large number of measurements at each substrate concentration and was not a practical proposition during the course of this work.

The second method was the plot of the ratio of substrate concentration to initial velocity versus substrate concentration (Hanes, 1932). This method was chosen since it is a procedure that has been shown to be best suited to analysis by the method of least squares (Henderson, 1978). An alternative plotting procedure, generally ascribed to Hofstee (1952), was not used since it involves a plot of initial velocity versus the ratio of initial velocity to substrate concentration. Such a method means that the dependent variable (initial velocity) lies on both axes of the plot and this results in an angular distortion of errors. Such distortion is not compatible with data-fitting by the least-squares criterion (Ferdinand, 1976; Henderson, 1978).

Clearly, if the replotted data fitted perfectly to the equation of the rectangular hyperbole (Dixon and Webb, 1979) each plot would yield the same  $K_m$  and  $V$  values. However in practice the errors in the dependent variable may be distorted differently in each plot. In this case each plot might give a different answer. Since the vast majority of the literature contains estimates of  $K_m$  and  $V$  based on unweighted fits to the Lineweaver-Burk double reciprocal plot, then, for comparison, this method was shown in the results. The procedure of Hanes was also used and, where differences between the respective determined  $K_m$  and  $V$  values existed, this latter method was considered to give the more reliable results.

#### 4.4.2.2 UDP-dependent initial reaction velocities

The initial experiment, which utilised a fairly wide range of variable UDP concentrations, indicated that the apparent  $K_m$  lay around 0.61mM. This value was determined from the single reciprocal plot. When the experiment was repeated over a narrower range of substrate concentration, the apparent  $K_m$  was slightly lower and it was proving difficult to obtain reasonable estimations of initial velocities at concentrations of UDP less than the calculated  $K_m$  levels. When the range of UDP in the digest was kept below 1mM, plots of initial velocity versus substrate concentration showed a

sigmoidal response and attempts to linearise the data proved unsuccessful. This result seems to suggest that at concentrations of UDP less than 0.4mM, the enzyme may have been responding in a co-operative manner. Murata (1972) has reported a similar phenomenon for UDP-dependent initial reaction velocities estimated for rice grain sucrose synthase, however Nomura and Akazawa (1973), in a more detailed study of this reaction using rice grain sucrose synthase, obtained no evidence for sigmoidal kinetics. Pontis and Salerno (1982) have demonstrated that sucrose synthase preparations from wheat embryos exhibited sigmoidal kinetics, however this was shown to be due to a protein factor which they isolated from whole wheat grains. The data obtained during the course of the present work show that concentrations of UDP in excess of 0.6mM readily overcame any apparent lag phase in the UDP-dependent reaction velocities of wheat endosperm sucrose synthase. However the estimations of endosperm UDP concentrations, shown previously in section 3.4.2, ranged between 0.08 and 0.24mM. While these estimations were minimal, i.e. they do not take into account possible compartmentalisation effects, they do suggest that UDP may be rate-limiting. Previously (section 4.4.1), it was pointed out that the in vitro determinations of sucrose synthase activity in developing endosperms suggested an excess of catalytic activity. In view of the data discussed in this section, it is possible that the in vivo levels of sucrose synthase activity may be rate-limiting with respect to conversion of sucrose to starch. The regulation of that activity might depend upon the generation of UDP. There may also be a role for ADP-dependent sucrose synthase activity in wheat endosperms, particularly since ADP was found to be present at higher levels than UDP (section 3.5). During the course of the present work attempts were made to estimate ADP-dependent sucrose synthase activity, however it was found that the adenosine moiety in AMP, ADP and ADP-glucose interfered with the Nelson reagent (data not shown). This prevented analysis of ADP-dependent sucrose synthase activity. For future work on this enzyme assay procedures should avoid the reducing sugar method of analysis (section 4.2.5). An alternative procedure might be a radiochemical assay which would



utilise  $[U-^{14}C]$  sucrose in the formation of either ADP-  $[U-^{14}C]$  glucose or UDP-  $[U-^{14}C]$  glucose (Su and Preiss, 1978). The assay procedure of Morell and Copeland (1985), which utilises UDP-glucose dehydrogenase, is only suitable for determination of UDP-dependent sucrose synthase activity.

#### 4.4.2.3 Sucrose-dependent initial reaction velocities

The calculated  $K_m$  (sucrose) for sucrose-dependent endosperm sucrose synthase was approximately 165mM and was determined over both broad and narrow concentration ranges. This  $K_m$  (sucrose), was of an order approaching 300 times that determined for  $K_m$  (UDP) in the same reaction and this ratio of 300 was similar to the ratio of amounts of sucrose and UDP per endosperm respectively which can be calculated from the data shown previously in section 3.3.1. The relatively high  $K_m$  for sucrose has been reported to be a feature of sucrose synthases extracted from a variety of plant tissues (Avigad, 1982).

$K_m$  has been defined previously (section 4.2.12) as the substrate concentration at 50 per cent of maximum velocity, and this implies that the concentration of sucrose necessary to achieve maximum velocity may be at least as high as 330mM. This value is approximately 6 times the estimated endosperm sucrose concentration (section 3.4.2) and suggests that the apparent excess catalytic activity, measured during endosperm development (section 4.4.1), may be necessary to alleviate the relatively high  $K_m$  (sucrose). This of course assumes that in vitro enzyme activities properly reflect the catalytic efficiency within intact tissue.

#### 4.4.3 Endogenous inhibitor studies

There was no significant evidence to suggest that either relatively low enzyme activities during the early stages of development or dropping levels in the later stages were due to the presence of endogenous inhibitors. This implies that the varying levels of sucrose synthase activity, observed during endosperm development,

may have been a function of specific rates of synthesis and degradation of that enzyme and would be consistent with a role for sucrose synthase as a developmental enzyme.

#### 4.5 Conclusions

Although sucrose synthase activity during wheat endosperm development was measured in apparent excess of the minimum catalytic requirement, the determined  $K_m$  values (UDP and sucrose respectively) suggest that the in vivo operating rate of the enzyme may be a good deal lower than the in vitro determinations. This conclusion was made because the previously determined levels of UDP and sucrose per endosperm relate to estimated respective endosperm concentrations (section 3.4.2) that were substantially less than the calculated  $K_m$  values (UDP and sucrose) for the cleavage of sucrose.

While the evidence for the observed sigmoidal curve of initial reaction velocity (UDP-dependent) versus substrate concentration was limited to one set of experimental data, the determinations of the same parameter at the 2 wider ranges of UDP concentration confirmed barely detectable levels of activity at concentrations of UDP less than the calculated  $K_m$ . Cooperative or allosteric phenomena might be consistent with an oligomeric structure for sucrose synthase. Such features have been demonstrated in sucrose synthase from rice grains (Nomura and Akazawa, 1973) and maize kernels (Su and Preiss, 1978) although in both instances there was no dissection of maternal and embryonic tissues prior to preliminary homogenisation procedures.

The pattern of sucrose synthase activity during endosperm development, the potential rate limiting concentrations of UDP and sucrose together with preliminary evidence of cooperative phenomena suggest that sucrose synthase may function in the regulation of starch biosynthesis in wheat endosperms. The mode of regulation might be directed at the level of protein biosynthesis (coarse



control) or more specifically by allosteric modulation of enzyme activity.

A role for invertase in the regulation of starch biosynthesis seems unlikely, although the data determined in the current work suggest that this enzyme has the capacity for a contribution to dry weight accumulation. However, as was concluded previously (section 3.5), invertase activity might be more crucial in the early days of endosperm development when the levels of UDP and sucrose synthase are low or limiting. Clearly in this situation, hydrolysis of sucrose to its consistent hexose sugars would be a significant factor in endosperm development.

## CHAPTER FIVE

5.0 ADP- and UDP-glucose pyrophosphorylases in developing wheat endosperms5.1 Introduction

Previously (section 4.4), increasing sucrose synthase activity in developing endosperms was observed to correlate with the onset of rapid endosperm dry weight accumulation. However the apparent surfeit of enzyme activity, as estimated in vitro, may not be realised in vivo. This conclusion was made on the basis of metabolite estimations in developing wheat endosperms and with respect to a study of sucrose synthase initial reaction velocities under substrate limiting conditions. If the catabolism of sucrose in wheat endosperms is limited by substrate availability (sucrose and UDP) then sucrose synthase may be involved in the regulation of endosperm starch biosynthesis.

ADP-glucose pyrophosphorylase has been strongly advocated in respect of the regulation of starch biosynthesis (Preiss, 1982a). However most of the evidence derives from studies on this enzyme as extracted from leaf or bacterial tissues. Very little data exists to implicate this enzyme in the regulation of cereal endosperm starch biosynthesis. Dickinson and Preiss (1969a and 1969b) have studied the activity of ADP-glucose pyrophosphorylase from homogenates of whole maize kernels. Clearly, since these homogenates contain tissues of differing genetic origins (pericarp, embryo and endosperm) it is invalid to assign mixed enzyme activities to a sole tissue i.e. the endosperm. Similarly, ADP-glucose pyrophosphorylase has been investigated in preparations from whole wheat flour (Espada, 1962; Tovey and Roberts, 1970) but not in developing wheat endosperms. Since there are clear morphological and physiological distinctions between chlorophyllous and non-chlorophyllous plant tissue, it cannot be assumed that the

mechanism of starch metabolism will be the same in both tissues. Consequently a major aim of this work was to investigate the nature of both leaf and endosperm ADP-glucose pyrophosphorylases from the same wheat cultivar i.e. cv. Sicco.

UDP-glucose pyrophosphorylase has been implicated in the P<sub>PPi</sub>-dependent catabolism of UDP-glucose to form G1P and UTP and it has been suggested that this UDP-glucose dependent production of G1P forms substrate for the ADP-glucose pyrophosphorylase reaction (Turner, 1969; Turner and Turner, 1975). If ADP- and UDP-glucose pyrophosphorylases were both located in the same cellular compartment then this type of metabolism may be wasteful in terms of the requirement for protein biosynthesis. However separation of the two activities by a membrane barrier, e.g. the amyloplast membrane, might provide a system of metabolic regulation of starch biosynthesis, possibly linked to the availability of P<sub>PPi</sub>. In the current work the levels of endosperm UDP-glucose pyrophosphorylase in developing endosperms have been determined and these levels are discussed in relation to sucrose synthase and ADP-glucose pyrophosphorylase activities from the same tissue.

## 5.2 Methods

### 5.2.1 Plant material

Plant material was grown and sampled as described in section 3.2.1.

### 5.2.2 Reagents

Alkaline phosphatase (EC 3.1.3.1) type III from E. Coli; glucose-6-phosphate dehydrogenase (EC 1.1.1.49) type IX from bakers yeast; glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.22) from bakers yeast; hexokinase (EC 2.7.1.1) type C301 from bakers yeast; inorganic pyrophosphatase (EC 3.6.1.1) from bakers yeast; phosphoglucomutase (EC 2.7.5.1) from rabbit muscle; phosphoglucose isomerase (EC 5.3.1.9) type X from bakers yeast; 3-phosphoglycerate

dehydrogenase (EC 1.1.1.95) from chicken liver; phosphoglycerate kinase (EC 2.7.2.3) from bakers yeast and phosphorylase-a (EC 2.4.1.1) from rabbit muscle were obtained from the Sigma Chemical Co. Ltd., Poole, Dorset.

Adenosine diphospho -D- [U- $^{14}\text{C}$ ] glucose-ammonium salt (ADP - [U- $^{14}\text{C}$ ] glucose);  $\alpha$  -D- [U -  $^{14}\text{C}$ ] glucose-1-phosphate-potassium salt ( [U -  $^{14}\text{C}$ ] G1P) and uridine diphospho-D- [U -  $^{14}\text{C}$ ] glucose-ammonium salt (UDP - [U -  $^{14}\text{C}$ ] glucose) were purchased from Amersham International plc., Amersham, Buckinghamshire.

Whatman DEAE-cellulose ion exchange chromatography paper (catalogue no. DE 81-3658 915) size 47 x 57cm and Whatman 1 CHR chromatography paper (catalogue no. 3001 917) size 46 x 57cm were supplied by McKay and Lynn, Edinburgh.

The following scintillation grade chemicals were obtained from Koch-Light Ltd., Maidstone, Kent; 2-phenyl-5- (4-biphenyl)-1,3,4-oxadiazole (PBD); 1,4-di-(2-(5-phenyl oxazolyl))-benzene (POPOP) and toluene.

All other reagents were as previously listed (sections 3.2.2 and 4.2.2) or of analytical grade or of the highest purity available.

### 5.2.3 Tissue extracts for enzyme studies

Endosperm extracts were prepared in ice-cold buffer essentially as described in section 4.2.3 except that the homogenisation buffer was composed of (mM): MOPS, pH 7.0, 50; dithioerythritol (DTE), 5; ATP, 0.2;  $\text{MgCl}_2$ , 10 and 2% w/v glycerol.

For large scale preparations, 25 day old endosperms (approx. 400 with a combined fresh weight of about 10g) were dissected free of pericarp/testa and embryo tissues and placed immediately into a pre-weighed glass beaker containing ice-cold homogenisation buffer (25ml) as described above. Following addition of the endosperms the

beaker was reweighed and the entire contents transferred to a hand-held all-glass homogeniser and homogenised on ice as previously described for the smaller scale preparations (section 4.2.3). The homogenate was centrifuged as detailed in section 4.2.3 and the final supernatant was analysed immediately for enzyme activity prior to utilisation for enzyme purification.

Extracts of leaf tissue were prepared from flag leaves (fresh weight about 60g) cut from young plants just prior to ear emergence. Flag leaves showing signs of senescence were discarded. The leaves were cut up into small pieces (approx. 1.5cm long) and placed in ice-cold homogenisation buffer (450ml). The preparation was then homogenised in an Osterizer Dual Range Pulse-Matic 16 homogeniser (1 min x 5) with cooling intervals (1 min) when the homogeniser bowl was placed in a bucket of ice. The resulting homogenate was centrifuged for 15 min at 2°C and 10 000g ( $r_{av} = 7.5\text{cm}$ ) in an MSE High Spin 25 centrifuge. The final supernatant was analysed immediately for enzyme activity prior to enzyme purification.

#### 5.2.4 Enzyme assays

##### 5.2.4.1 ADP-glucose pyrophosphorylase (EC 2.7.7.27), G1P-dependent activity

Nucleoside diphosphate sugar formation was assayed essentially by the method originally described by Shen and Preiss (1964) which was an adaptation of a radioactive ion-exchange procedure devised by Sherman (1963). The assay measured the incorporation of  $[U-^{14}C]$  glucose from  $[U-^{14}C]$  G1P into ADP -  $[U-^{14}C]$  glucose and is summarised by the following reaction:



The enzyme digests were composed of (mM):  $[U-^{14}C]$  G1P (0.74  $\mu\text{Ci } \mu\text{mole}^{-1}$ ), 0.68; ATP, 1.20;  $\text{Mg Cl}_2$ , 5; HEPES (pH 8.0),

100, inorganic pyrophosphatase, 0.625 units (Sigma) and distilled water (5–45  $\mu$ l depending upon the activity of the initiating enzyme). Digests, with and without ATP, were incubated in a water bath at 25°C prior to (5 min) and for the duration of the assays which were initiated by the addition of enzyme extracts (5–45  $\mu$ l). The total digest volumes, including enzyme extracts were 100  $\mu$ l. Following initiation of the reaction the assays were terminated, after varying lengths of time (0–20 min), by heating in a boiling water bath for 45s. On cooling, alkaline phosphatase (0.5 units (Sigma)) was added to each digest in order to cleave the phosphate moiety from any unreacted  $[U-^{14}C]$  G1P and, following incubation for 1.5h in a water bath at 25°C, aliquots (100  $\mu$ l) of the alkaline phosphatase treated mixtures were spotted onto respective DEAE-cellulose paper strips (1.5 x 8cm). The strips were dried in an ambient air flow and then swirled in distilled water (600ml x 3) for a total of 3 min. Following swirling, the strips were again dried in an ambient air flow before being counted in scintillation vials containing toluene-based scintillation cocktail (5ml) composed of (mM): PBD, 30.2 and POPOP, 1.4. Results were expressed in counts per min (cpm) and, where appropriate, corrected to disintegrations per min (dpm) using quenching and label retention data.

### Assay controls

#### Quenching

Enzyme digests were prepared essentially as described above except that non-radioactive G1P was utilised instead of the labelled substrate. At the conclusion of the digest, aliquots (100  $\mu$ l), of the alkaline phosphatase-treated mixture were spotted onto the DEAE-cellulose strips. The strips were then dried, swirled in distilled water, and redried as previously described. Following redrying, aliquots (8  $\mu$ l) of standard ADP-  $[U-^{14}C]$  glucose (12.5  $\mu$  Ci ml<sup>-1</sup>) and UDP-  $[U-^{14}C]$  glucose (12.5  $\mu$  Ci ml<sup>-1</sup>) and an aliquot (20  $\mu$ l) of standard  $[U-^{14}C]$  G1P (5  $\mu$  Ci ml<sup>-1</sup>) were

spotted onto respective dried strips. The strips were then counted in scintillation vials containing scintillation cocktail as detailed above. Each standard was evaluated in triplicate and the efficiencies of counting calculated as a percentage of the known count rate (dpm).

#### Label retention

The percentage retention of the labelled nucleoside diphosphate sugars by the DEAE-cellulose paper strips and the efficiency of  $[U-^{14}C]$  G1P elution were calculated as follows:

ADP-  $[U-^{14}C]$  glucose, UDP-  $[U-^{14}C]$  glucose and  $[U-^{14}C]$  G1P were incubated in the manner described for the ADP-glucose pyrophosphorylase assay except that the digest component volumes were doubled and a heat-inactivated enzyme extract was substituted for the active extract. For retention assays of the labelled nucleoside diphosphate sugars,  $[U-^{14}C]$  G1P was substituted by aliquots (90  $\mu$ l) of either ADP-  $[U-^{14}C]$  glucose (1.1  $\mu$  Ci ml<sup>-1</sup>) or UDP -  $[U-^{14}C]$  glucose (1.1  $\mu$  Ci ml<sup>-1</sup>). The digest concentrations of both nucleoside diphosphate sugars were 0.41mM. Assays were effected in triplicate, with and without alkaline phosphatase and with and without swirling. Results were expressed as a percentage of the unswirled count, thus enabling the calculation of retention and elution percentages for the 3 labelled compounds.

Each fresh batch of alkaline phosphatase was dialysed overnight at 4°C against 100mM HEPES buffer at pH 8.0 containing DTE (5mM) prior to incorporation in the assay. This procedure was essential since the enzyme preparation was suspended in 2.5M (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> solution and this high salt concentration interferes with the ion-exchange procedure.

#### 5.2.4.2 ADP-glucose pyrophosphorylase, P<sub>Pi</sub>-dependent activity

The formation of G1P was assayed by a procedure adapted from Espada (1966).

The amount of G1P formed by incubation of ADP-glucose with P<sub>Pi</sub> together with the enzyme extract was determined by adding phosphoglucomutase, glucose-6-phosphate dehydrogenase and NADP. The reduced NADP was then estimated by measuring the increase in absorbancy at 340nm. The digest was composed of (mM): ADP-glucose, 2; MgCl<sub>2</sub>, 5; HEPES (pH 8.0), 100; DTE, 2; NaF, 10; glucose-1, 6-bisphosphate, 0.02; NADP, 0.6; Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> · 10 H<sub>2</sub>O (P<sub>Pi</sub>), 1.5; phosphoglucomutase, 1 unit; glucose-6-phosphate dehydrogenase, 2.9 units; distilled water (0.19ml) and enzyme extract (0.1ml). The total digest volume was 1ml. The digest substrates and cofactors, excluding P<sub>Pi</sub>, were carefully mixed in a Sarstedt 10 x 4 x 45mm acrylic cuvette and allowed to incubate for 3 min at 25°C. Following incubation, the coupling enzymes were added, mixed and incubated for a further 1 min. Enzyme extract was then added, mixed and allowed to incubate for 1 min or until the absorbancy reading became steady. Finally the reaction was initiated by the addition of P<sub>Pi</sub>. The reaction was monitored continuously at 25°C in a Pye Unicam SP 8-100 UV/Vis recording spectrophotometer at 340nm. Control assays in the absence and presence of ADP-glucose and P<sub>Pi</sub> respectively and using boiled enzyme blanks were carried out. Additionally the assay function was tested using exogenous G1P.

#### 5.2.4.3 UDP-glucose pyrophosphorylase (EC 2.7.7.9), G1P-dependent activity

The formation of UDP-glucose was assayed as described for ADP-glucose pyrophosphorylase in section 5.2.4.1 except that UTP (1.20mM) was substituted for ATP in the enzyme digest. All other conditions were identical.



#### 5.2.4.4 Control enzyme assays

The following enzymes were assayed to establish the presence of contaminating enzymes that may have been present in extracts of endosperm and leaf tissue being used for studies on ADP- and UDP-glucose pyrophosphorylases. These contaminating enzymes were generally analysed under their respective published optimum conditions. Where commercial preparations were available (see section 5.2.2) these were used to test the validity of the assay method. Control assays were carried out using boiled endosperm and leaf tissue extracts and using active preparations but excluding substrate(s). In general, duplicate analyses of the extracts were carried out. Where certain activities could not be detected the assay was repeated a further 2 times.

#### Hexokinase

Glucose- and fructose-dependent hexokinase activities were measured by an adaptation of the method previously described in section 3.2.4.1. The enzyme digest was composed of (mM): glucose or fructose, 0.24; triethanolamine (pH 7.6), 50; NADP, 1;  $\text{Mg SO}_4$ , 10; ATP, 1; glucose-6-phosphate dehydrogenase, 2.89 units (Sigma); distilled water, 1.86ml and enzyme extract, 0.02ml in a total digest volume of 3ml. The digest components, excluding the sugar substrate, were incubated for 5 min at 25°C in a Sarstedt 10 x 10 x 45mm acrylic cuvette. Following incubation, the sugar substrate was added, the contents thoroughly mixed and the absorbance at 340nm monitored as described for the reverse ADP-glucose pyrophosphorylase assay in section 5.2.4.2.

#### Phosphoglucose isomerase

Phosphoglucose isomerase activity was measured as described for hexokinase activity except that the initiating substrate was F6P (0.14mM in digest), instead of glucose or fructose.

Phosphoglucomutase

Phosphoglucomutase activity was measured as described for hexokinase activity except that the initiating substrate was GlP (0.08mM in digest) instead of glucose or fructose).

Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase activity was measured as described for hexokinase activity except that glucose-6-phosphate dehydrogenase was omitted from the digest and the initiating substrate was G6P (0.14mM in digest) instead of glucose or fructose.

Alkaline phosphatase

Alkaline phosphatase activity in enzyme extracts was measured essentially as described by Bergmeyer (1974a). The digest was composed of (mM): HEPES (pH 8.0), 100;  $MgCl_2$ , 1; p-nitro phenyl phosphate, 6 and distilled water to 2.98ml. The digest was incubated at 25°C for 5 min and the reaction initiated by the addition of enzyme extract (0.02ml). Following initiation, the reaction was monitored by following the increase in absorbance at 400nm in a Pye Unicam SP 8-100 UV/Vis recording spectrophotometer.

Nucleoside diphosphate kinase (EC 2.7.4.6)

Nucleoside diphosphate kinase activity in enzyme extracts was assayed by the method of Baxter (1972). The assay monitored the following reaction:



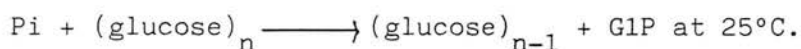
The reaction was measured in the direction of ATP synthesis and was coupled to the hexokinase and glucose-6-phosphate dehydrogenase reactions already described. The enzyme digest was composed of

(mM): ADP,1; UTP,1;  $\text{MgCl}_2$ , 2; HEPES (pH 8.0), 50; glucose, 10; NADP, 1; distilled water, 0.56ml and hexokinase/glucose-6-phosphate dehydrogenase (Boehringer Mannheim see section 3.2.4), 0.02ml. The reaction was initiated by the addition of enzyme extract (0.02ml). The total digest volume was 1ml. The course of the reaction was monitored continuously as previously described for the hexokinase assay.

### Phosphorylase

Phosphorylase activity in enzyme extracts was measured by a modified procedure developed from Bergmeyer (1974b).

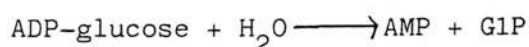
The assay measured the following reaction:



The G1P was then measured by the coupled reaction of phosphoglucomutase and glucose-6-phosphate dehydrogenase as described for the phosphoglucomutase assay. The assay digest was composed of (mM): NADP, 0.34;  $\text{MgCl}_2$ , 5; HEPES (pH 8.0), 100;  $\text{KH}_2\text{PO}_4$  (Pi), 10; wheat starch ( a stock solution of  $25 \text{ mg ml}^{-1}$  was made up in water and centrifuged to give a clear supernatant),  $2 \text{ mg ml}^{-1}$ ; phosphoglucomutase, 30 units (Sigma); glucose-6-phosphate dehydrogenase, 2.89 units (Sigma); distilled water, 1.5ml and enzyme extract, 0.02ml. The digest components, including enzyme extract but excluding  $\text{KH}_2\text{PO}_4$  and wheat starch, were incubated at  $25^\circ\text{C}$  for 5 min. Following initiation with Pi and wheat starch the reaction was monitored as described for the phosphoglucomotase assay.

### ADP-glucose pyrophosphatase (EC 3.6.1.21)

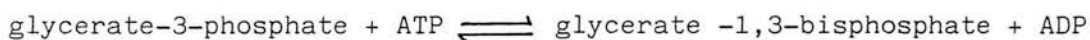
This assay measured the following reaction:



The formation of G1P was then measured using the phosphoglucomutase/glucose-6-phosphate dehydrogenase reaction as described above. The digest was composed of (mM): ADP-glucose, 1.33; triethanolamine (pH 7.6), 50; NADP, 1;  $\text{MgCl}_2$ , 5; distilled water, 0.55ml; glucose-6-phosphate dehydrogenase, 2.89 units; phosphoglucomutase, 30 units and enzyme extract (0.02ml) in a total digest volume of 1.5ml. The assay mixture, excluding ADP-glucose, was equilibrated at 25°C and initiated with the addition of ADP-glucose. The rate of the reaction was monitored as for the phosphorylase assay.

#### Phosphoglycerate kinase

Phosphoglycerate kinase activity in enzyme extracts was carried out essentially according to the method of Bergmeyer (1974c). The assay measured the following reaction:



The digest was composed of (mM) : HEPES (pH 8.0), 100; EDTA, 1; NADH, 0.2; ATP, 1.0; glycerate-3-phosphate, 6.5;  $\text{MgSO}_4$ , 1.67; distilled water, 0.305ml; glyceraldehyde-3-phosphate dehydrogenase, 4 units (Sigma) and enzyme extract (0.02ml). The total digest volume was 3ml. The digest components, excluding the enzyme extract and glycerate-3-phosphate were incubated at 25°C for 5 min. The enzyme extract (0.02ml) was then added and a further incubation of 1 min was carried out before the reaction was initiated by the addition of glycerate-3-phosphate. The resulting coupled reaction:



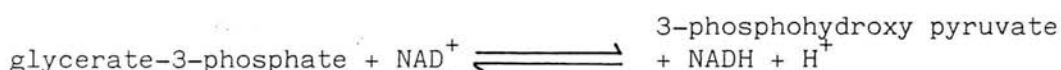
was followed by monitoring the decrease in absorbance at 340nm in a Pye Unicam SP 8-100 UV/Vis recording spectrophotometer.

### Glyceraldehyde-3-phosphate dehydrogenase

The presence of glyceraldehyde-3-phosphate dehydrogenase in enzyme extracts was measured essentially as described for the phosphoglycerate kinase assay except that glyceraldehyde-3-phosphate dehydrogenase was excluded and phosphoglycerate kinase (4 units, Sigma) was included.

### 3-Phosphoglycerate dehydrogenase

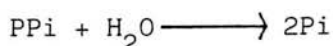
The activity of 3-phosphoglycerate dehydrogenase in enzyme extracts was measured according to the method of Sugimoto and Pizer (1968). The assay measured the following reaction:



and monitored the increased in absorbance at 340nm. The enzyme digest was composed of (mM): HEPES (pH 8.0), 50; NAD, 1; glycerate-3-phosphate, 10; DTE, 5; enzyme extract, 0.02ml and distilled water, 1.78ml. The total digest volume was 3ml. The reaction was initiated by the addition of glycerate-3-phosphate and followed at 25°C in a Pye Unicam SP 8-100 UV/Vis recording spectrophotometer.

### Inorganic pyrophosphatase

The measurement of inorganic pyrophosphatase activity in enzyme extracts was carried out using the methods described by Bergmeyer (1974d) and Ames (1966). The assay measured the following reaction:



The assay digest was composed of (mM): HEPES (pH 8.0), 50;  $\text{MgCl}_2$ , 5; PPi, 1.6; distilled water, 2.284ml and enzyme extract, 0.02ml. The total digest volume was 3ml. The digest excluding PPi, was

incubated for 5 min at 25°C before initiation of the reaction by the addition of PPI. Following mixing the digest was incubated at 25°C for 15 min and then assayed for phosphate.

The phosphate reagent was composed of (1) ascorbic acid (500mM) and (2) ammonium molybdate (3.4mM) in  $\text{H}_2\text{SO}_4$  (1M) mixed in the ratio of 1 part of (1) to 6 parts of (2).

On completion of the digest, aliquots (7ml) of the phosphate reagent were added to the digest tubes, thoroughly mixed and then incubated for 1h at 37°C. Following incubation, the tubes were measured for absorbance at 820nm and calibrated for phosphate against a suitable calibration curve prepared from standards in the range 0-0.10  $\mu$  moles phosphate per 10ml digest.

#### 5.2.5 Product identification

The nucleoside diphosphate sugars formed during the reaction (section 5.2.4.1) were identified directly by the following method (S.C. Fry personal communication):

[ $\text{U-}^{14}\text{C}$ ] glucose was incorporated into ADP- and UDP-glucose using essentially the assay methods detailed in sections 5.2.4.1/3 except that the digest volumes were doubled. Three assay digests were set up per enzyme extract for each nucleoside diphosphate sugar. For each set (3) of assay digests 3 DEAE-cellulose paper strips were spotted with 0.15ml of the alkaline phosphatase-treated mixture. The strips were dried, swirled in distilled water and redried in the usual manner. One strip per set of 3 was counted in scintillation cocktail as previously described (section 5.2.4.1) while the other 2 were individually coiled and placed in respective 2ml plastic syringes and pushed to the bottom by the syringe plunger. An Eppendorf tube was placed at the outlet of the syringe and the whole apparatus placed in a 10ml plastic centrifuge tube with the syringe flanges resting on the lip of the centrifuge tube.

Pyridine (5% v/v) - acetic acid buffer at pH 5.0 (0.5ml) was carefully pipetted onto the coiled strip. The apparatus was then centrifuged in an MSE bench centrifuge operating at 3 250rpm at room temperature for 10 min. Following centrifugation, the procedure was repeated using a further 0.2ml of the pyridine acetic acid buffer. The total eluate was then freeze dried overnight in an FTS Dura-Top bulk tray drier (FTS systems Inc., USA) and the resulting residue taken up respectively in 2ml of either non-radioactive ADP-glucose (3mM) or non-radioactive UDP-glucose (3mM). The spent paper strip was dried and then counted for non-eluted radioactivity and compared with the untreated strip for efficiency of elution. Portions of the freeze dried eluate were then subjected to analysis by paper electrophoresis chromatography. The following systems were utilised:

- (1) paper electrophoresis in pyridine/acetic acid buffer at pH 6.5 for 55 min at 3 000 volts.
- (2) paper electrophoresis in pyridine/acetic acid buffer at pH 3.5 for 55 min at 3 000 volts.
- (3) paper electrophoresis in pyridine/acetic acid buffer at pH 2.1 for 55 min at 3 000 volts.
- (4) descending paper chromatography in aqueous 71 per cent v/v ethanol containing  $\text{CH}_3\text{COONH}_4$  (290mM) and EDTA (0.08mM), previously pH adjusted to 7.0, for 48h.

Samples were run against radiolabelled standards (Amersham International) and calibrated against  $[\text{U-}^{14}\text{C}]$  G1P. In addition, co-chromatography was carried out using a mix of standard and freeze dried preparations. All chromatography i.e. paper electrophoresis and paper chromatography was effected using Whatman

No. 1 chromatography paper (effective separation length 40cm and 45cm with paper electrophoresis and paper chromatography respectively). All chromatograms were prepared in duplicate with one being sprayed with a phosphate indicator while the other was separated into lanes, cut into 1cm wide strips and counted in scintillation vials containing the previously described scintillation cocktail (section 5.2.4.1).

A further portion of the respective freeze dried residues was hydrolysed with 0.1M trifluoroacetic acid (1ml) by heating in a boiling water bath for 30 min. The hydrolysate was freeze dried as previously described and taken up in a solution (2ml) of non-radioactive glucose (11mM). The mixture was then chromatographed by descending paper chromatography in the manner previously described. The mobile phase was ethyl acetate - pyridine -  $H_2O$  (8:2:1). Various mono-saccharide standards were chromatographed in parallel lanes and the results calibrated against the mobility of glucose (R<sub>g</sub>).

The following reagents were used to visualise the chromatograms:

- (1) phosphate reagent;  $H_2O$  (12ml), conc. HCl (3ml), ammonium molybdate (1.6 m Moles in  $H_2O$  (5ml)) and 60% perchloric acid (6ml) all dissolved and then added to acetone (180ml).
- (2) carbohydrate reagent; p-anisidine HCl (45 m Moles) dissolved in butanol (160ml), ethanol (40ml) and distilled water (40ml) plus a trace of stannous chloride.

#### 5.2.6 Ammonium sulphate fractionation of endosperm extracts

Endosperm extracts derived from the large-scale preparations described in section 5.2.3 were subjected to fractionation by ammonium sulphate essentially according to the procedures extensively reviewed by Scopes (1984a).



An extract was estimated for volume using a measuring cylinder and a small aliquot (about 10ml) retained at 4°C in the refrigerator (at no time were any of the samples, pre- or post-fractionation, deep frozen). The remainder of the extract was fractionated in increments of 10 per cent up to 90 per cent.

The amount of ammonium sulphate required to be added to give the correct incremental increase in per cent saturation at the various stages of the fractionation procedure was calculated according to the following equation:

$$g = \frac{533 (S_2 - S_1)}{100 - 0.3S_2}$$

where  $g$  = required addition of ammonium sulphate in grams per litre of extract at 20°C.

$S_1$  = starting per cent saturation of ammonium sulphate

$S_2$  = required per cent saturation of ammonium sulphate

e.g. for 10 per cent ammonium sulphate  $S_1 = 0$  and  $S_2 = 10$   
 for 20 per cent ammonium sulphate  $S_1 = 10$  and  $S_2 = 20$  etc.

This formula takes into account the increase in volume due to ammonium sulphate addition i.e. 0.56ml per g of ammonium sulphate added.

Following careful addition and dissolution of the calculated amount of ammonium sulphate, the solution was stirred for 30 min at about 4°C in a refrigerator using a magnetic stirrer. The precipitate was then collected by centrifuging for 15 min at 2°C and 10 000 g ( $r_{av} = 7.5\text{cm}$ ) in an MSE High Spin centrifuge. The resultant supernatant was measured for volume and retained on ice. The pellet was resuspended in 2ml of homogenisation buffer (section 5.2.3) containing ammonium sulphate (85 per cent w/v) and stored in a

refrigerator at around 4°C. The supernatant, previously retained on ice, was then subjected to the next fractionation. In this way a series of pellets, precipitated as a function of increasing increments of 10 per cent ammonium sulphate, were obtained individually and stored separately in 85 per cent w/v ammonium sulphate solution at 4°C.

#### 5.2.7 Ammonium sulphate fractionation of leaf extracts

Leaf extracts derived from the procedures detailed in section 5.2.3 were subjected to ammonium sulphate fractionation in exactly the same manner as described for the enzyme extracts (section 5.2.6).

#### 5.2.8 Measurement of enzyme activity in ammonium sulphate fractions

The measurement of enzyme activities in the ammonium sulphate fractions was carried out by first dialysing an aliquot (0.1ml) of the stored fractions (section 5.2.6) against homogenisation buffer (section 5.2.3) at 4°C for 3h. This temperature was maintained by dialysing in a refrigerator using a magnetic stirrer to circulate the dialysis buffer. Following dialysis, the dialysate was assayed for enzyme activities as described in section 5.2.4. Protein was determined in the dialysate as previously described (section 3.2.3.5).

#### 5.2.9 Gel filtration chromatography

Endosperm extracts derived from large-scale preparations described in section 5.2.3 were fractionated by broad ammonium sulphate cuts, i.e. 0-15, 15-30, 30-60, and 60-75 per cent, essentially as detailed for narrow cuts in section 5.2.6. Fractions were measured for enzyme activity by the procedures outlined in section 5.2.8 and portions (1 or 2ml) of the 30-60 per cent pellet dialysate were applied to an LKB 2137 water jacketed column containing LKB Ultrogel AcA 34 (fractionation range 20 000 - 400 000 daltons) column. The column dimensions were 50 x 1.6cm and the total bed

volume was  $100.5\text{cm}^3$ . The void volume ( $V_0$  blue dextran) was 32ml and the elution volume ( $V_e$  potassium dichromate) was 90ml. The dilution factor for  $V_e$  was 13. The eluting buffer was 20mM HEPES at pH 8.0 which included 2-mercaptoethanol (20mM). The flow rate was  $22.5\text{ml h}^{-1}$  and the eluting buffer was pumped via a Watson Marlow HR Flow Inducer type 18 peristaltic pump. A cooling solution, consisting of ethanol (20% v/v) in water and maintained at  $< 2^\circ\text{C}$  by a cooling coil suspended in a Grant's bath, was pumped continuously through the water jacket of the column. Fractions (1.76ml) were controlled and collected by an LKB fraction collector comprising; (1) 3403 B controller, (2) 3428 drop counter, (3) 3402 B distributor and (4) 3401 turntable. The collecting tubes, mounted in the 3401 turntable, were immersed in an insulated cooling trough maintained at  $< 4^\circ\text{C}$  by a continuation of the cooling circuit described for the column jacket. The eluted fractions were monitored for soluble protein by measuring a sample of each fraction for absorbance at 280nm in a Pye Unicam SP 8-100 UV/Vis spectrophotometer. Each fraction was also measured for enzyme activity (ADP - glucose pyrophosphorylase) by the method described in section 5.2.4.2 for GlP formation. Active fractions were pooled, measured for volume and a sample (2ml) was dialysed against distilled water (2 x 200ml) prior to analysis for soluble protein by the method of Lowry as described in section 3.2.3.5. The remaining active eluate was concentrated to 60 per cent w/v with ammonium sulphate and stored in a refrigerator at  $4^\circ\text{C}$ .

A second buffer system was also utilised with the same column. The buffer components were (mM): triethanolamine (pH 8.0), 20; 2-mercaptoethanol, 10;  $\text{MgCl}_2$ , 10; ATP, 0.2 and glycerol, 2 per cent w/v. Chromatography conditions and eluate analyses were the same as for the HEPES system.

#### 5.2.10 Ion-exchange chromatography

Endosperm extracts derived from large-scale preparations as

described in section 5.2.3 were fractionated by narrow ammonium sulphate cuts as detailed in section 5.2.6. The fractions were measured for enzyme activity by the procedures outlined in section 5.2.8 and portions (1ml) of the 40 per cent pellet dialysate were applied to an LKB 2137 water jacketed column containing DEAE-cellulose. The column dimensions were 13.2 x 1.6cm and the total bed volume was 26.5cm<sup>3</sup>. A binary buffered gradient system was used to elute the enzymic protein and this system comprised (1) triethanolamine at pH 8.0 (20mM) containing (mM): DTE, 2; MgCl<sub>2</sub>, 10; ATP, 0.2; glycerol, 2 per cent w/v and (2) as 1 but with the inclusion of NaCl (400mM). The column was equilibrated with 4 column volumes of buffer 1 prior to application of the enzyme preparation. Three column volumes of buffer 1 were run through before starting the gradient at 1.2mM ml<sup>-1</sup>. Eluate was collected in 4 ml fractions by the fraction collecting system described in 5.2.9 and each fraction was assayed immediately for ATP- and UTP-dependent pyrophosphorylase activity in the direction of nucleoside diphosphate sugar formation as described in section 5.2.4.1/3. Fractions were also analysed for the presence of protein as detailed in section 5.2.9. The eluant flow rate was 0.2ml min<sup>-1</sup> and the total run time was 35h. At fraction 83, when 332ml of the eluate had been collected, the buffer was made 1.0M with respect to NaCl in order to elute any remaining protein. Throughout the run the column and the eluted fractions were kept cool by the same system as detailed in section 5.2.9.

#### 5.2.11 Enzyme studies on ADP- and UDP-glucose pyrophosphorylases from endosperm and leaf tissue

All the studies detailed in the remaining methods sections were carried out on the ammonium sulphate fractions described in sections 5.2.6/7 which were prepared for analyses as detailed in section 5.2.8. For endosperm enzyme studies the 40 per cent cut was utilised and for leaf enzyme studies the 50 per cent cut was employed. Enzyme activities were analysed in the direction of nucleoside diphosphate sugar formation as detailed in sections

5.2.4.1/3 and the controls were as described in these sections.

#### 5.2.11.1 pH profiles

The pH-dependent activities of endosperm and leaf ADP-glucose pyrophosphorylases and endosperm UDP-glucose pyrophosphorylase were determined as follows:

A series of overlapping buffers covering a range of pH values were prepared. The buffers (200mM) were composed of (pH): MES, 5.0, 5.5, 6.0, 6.5, 7.0; HEPES, 6.5, 7.0, 7.5, 8.0, 8.5 and glycine/NaOH, 8.0, 8.5, 9.0, 9.5, 10.0. Aliquots (50  $\mu$  l) of these buffers were substituted for HEPES (pH 8.0) buffer which was previously described for the assay (sections 5.2.4.1/3).

Enzyme reactions were initiated by addition of aliquots of enzyme extract (endosperm, 5  $\mu$  l and leaf, 10  $\mu$  l) and the digest times were 10 min and 15 min for the endosperm and leaf extracts respectively. Analyses were carried out in duplicate at each pH value.

#### 5.2.11.2 Reaction linearity

The linearity of the reaction rates for both endosperm ATP- and UTP-dependent pyrophosphorylase activity and for leaf ATP-dependent activity was measured essentially as described in sections 5.2.4.1/3. Aliquots (endosperm, 5  $\mu$  l and leaf, 10  $\mu$  l) were analysed over time periods of 20 min with sampling every 2.5 min for the endosperm extract and every 4.0 min for the leaf extract. Additionally the leaf extract activity was measured in the presence of 1mM glycerate-3-phosphate (3-PGA). UTP-dependent activity of the endosperm extract was measured in a similar manner as for the ATP-dependent activity.

#### 5.2.11.3 Endosperm ADP-glucose pyrophosphorylase activity as a function of time after dialysis

The activity of the endosperm 40 per cent ammonium sulphate dialysate was measured as a function of time after dialysis. Samples were analysed in the usual manner (section 5.2.4.1) with the dialysate kept at 4°C in a refrigerator between assays. Triplicate analyses were carried out at the following times (h after dialysis): 0, 3, 6, 9, 24.6 and 27. The dialysate aliquot for analysis was 5  $\mu$ l and the digest time was 10 min.

#### 5.2.11.4 Thermal characteristics of the endosperm enzyme

The heat-labile activity (ATP- and UTP-dependent) of the endosperm enzyme was determined by incubating the enzyme at various temperatures (25, 40, 55, 70 and 85°C) for increasing lengths of time (5, 10 and 15 min) before assaying the activity as previously described (sections 5.2.4.1/3). All assays were carried out in duplicate.

#### 5.2.11.5 Enzyme kinetics

Values for the concentrations of the substrates (G1P and ATP) required to give initial reaction rates at 50 per cent of the apparent maximum velocity, were determined for endosperm and leaf ADP-glucose pyrophosphorylases. In the case of the leaf enzyme, the effect of the presence of 3-PGA at varying concentrations in the assay digest was also investigated.

#### G1P-dependent initial reaction velocities

The assay digest was essentially as previously described (section 5.2.4.1) except that G1P was varied from 0 - 1.40mM for the endosperm extract and from 0 - 0.56mM for the leaf enzyme. The latter enzyme was assayed in the absence and presence of 3-PGA (1 mM). All other digest components were as previously described

(section 5.2.4.1).

#### ATP-dependent initial reaction velocities

The concentration of ATP in the assay digest (section 5.2.4.1) was varied from 0 - 1.0 mM for both endosperm and leaf enzymes. The latter enzyme was assayed in the absence and presence of 3-PGA (1mM). All other digest components were as previously described (section 5.2.4.1).

#### 3-PGA dependent initial reaction velocities

3-PGA in the assay digests for the leaf enzyme was varied from 0 - 1.0 mM. All other digest components were as previously described (section 5.2.4.1).

Enzyme extract aliquots and digest times for G1P-, ATP- and 3-PGA-dependent initial reaction velocities were as follows (  $\mu$  l, min): endosperm, 5, 10; leaf (G1P and ATP), 10, 15 and leaf (3-PGA), 10, 10. The assay digests were carried out in triplicate for the endosperm extracts, with and without 3-PGA (1mM) for the leaf G1P- and ATP-dependent initial reaction velocities and in duplicate for the leaf 3-PGA-dependent initial reaction velocities. Controls were as previously described (section 5.2.4.1).

The data for G1P- and ATP- dependent initial reaction velocities were plotted as previously described (section 4.2.12).

#### 5.2.11.6 Metabolite activation/inhibition

The effects of the following metabolites, on the activity of ADP-glucose pyrophosphorylases from endosperm and leaf tissue, were determined: 3-PGA, 2-PGA, GAP, 2-deoxyglucose, pyruvate, PEP, F-2, 6-bisP, F-1, 6-bisP, G-1, 6-bisP, malic acid, G6P, DHAP, lactic acid, glutamine, sucrose, glutamic acid, AMP, EDTA,  $\beta$ -G1P, UDP, ADP,  $\alpha$  KG, citric acid, CDP, ADP-glucose, UDP-glucose, PPI, Pi,

F6P, NADP, GDP, ascorbic acid and phytic acid.

All the above metabolites were included at a concentration of 1mM in individual assay digests, the components and conditions of which were essentially as previously described in section 5.2.4.1. The extract aliquots and digest times were as detailed in section 5.2.11.5.

#### 5.2.11.7 The effect of ADP-glucose, PPi and Pi on the activity of endosperm ADP-glucose pyrophosphorylase

The activity characteristics of the endosperm enzyme were investigated in the presence of ADP-glucose, PPi and Pi which were added individually to the assay digest through a concentration range of 0 - 4mM. Other digest components and conditions were as previously described (section 5.2.4.1) except that inorganic pyrophosphatase was excluded for the experiments with PPi in the digest. Assay digests were carried out in duplicate with a digest time of 10 min. The enzyme extract aliquots were 5  $\mu$  l.

#### 5.2.11.8 The effect of $\text{MgCl}_2$ , BSA, and DTE on endosperm ADP-glucose pyrophosphorylase activity

The activity characteristics of the endosperm enzyme in response to varying concentrations of  $\text{MgCl}_2$ , BSA and DTE were examined. Endosperm extracts (5  $\mu$  l) were assayed essentially as previously described (section 5.2.4.1) except that the above components were included individually through a range of concentrations which were as follows:  $\text{MgCl}_2$ , 0 - 25 mM; DTE, 0 - 25 mM and BSA, 0 - 0.4 mg  $\text{ml}^{-1}$ . Additionally  $\text{MgCl}_2$  and DTE were included at 5mM in all the digests except where the specific effect of either component was studied. In these cases the previously stated concentration ranges were applicable.

Following these initial studies, the effect of  $\text{MgCl}_2$  was re-investigated keeping DTE constant at 5 mM but varying  $\text{MgCl}_2$  from



0 - 4mM.

All enzyme digests were analysed in duplicate over a 10 min time period.

#### 5.2.11.9 Effect of various salts on endosperm ADP-glucose pyrophosphorylase activity

Aliquots (10  $\mu$  l) of the following salt solutions were added to portions (40  $\mu$  l) of endosperm extract which had been dialysed free of ammonium sulphate:  $\text{Na}_2\text{HPO}_4$  (20mM),  $\text{MgCl}_2$  (20mM),  $\text{MgCl}_2/\text{Na}_2\text{HPO}_4$  (10/10 mM) and NaCl (20mM). An aliquot (10  $\mu$  l) of distilled water was added to a fifth dialysate portion. 10  $\mu$  l of this water control was analysed immediately for ADP-glucose pyrophosphorylase activity (assay digest time was 10 min) and the 5 samples were then kept at 4°C in a refrigerator. After 16h, all 5 samples were analysed for ADP-glucose pyrophosphorylase activity essentially as described in section 5.2.4.1 and as detailed above for the initial water control assay.

A second experiment was set up as follows:

Aliquots (50  $\mu$  l) from the endosperm 40 per cent ammonium sulphate fraction were dialysed overnight against respective homogenisation buffers (section 5.2.3) containing (mM):  $\text{Na}_2\text{HPO}_4$ , 10;  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ , 10;  $(\text{NH}_4)_2\text{SO}_4$ , 10 and  $(\text{NH}_4)_6\text{MoO}_{24} \cdot 4\text{H}_2\text{O}$ , 10. A control dialysis against homogenisation buffer only was also carried out. Following dialysis each dialysate was diluted 3 times with its respective buffer and estimated for ADP-glucose pyrophosphorylase activity over a time course of 48 h. These diluted dialysates were maintained at 4°C in a refrigerator between assay times. All assays were carried out in duplicate as described in section 5.2.4.1. The dialysate aliquot for analysis was 5  $\mu$  l and the digest time was 10 min.

### 5.3 Results

### 5.3.1 The radiochemical assay ( [ U - $^{14}\text{C}$ ] ) for ATP- and UTP-dependent pyrophosphorylases

The determination of counting efficiencies and the retention and elution efficiencies of the strip-counting procedures are given in tables 5.1 and 5.2. These parameters, together with data supplied by Amersham International for the [ U -  $^{14}\text{C}$  ] radiochemicals, were incorporated in equations that were used to convert cpm to units of enzyme activity. These equations are given in table 5.3.

Table 5.1 Quenching and counting efficiencies

radiochemical standards (Amersham)	theoretical count (dpm x $10^{-5}$ )	quenched count* (cpm x $10^{-5}$ )	counting efficiency (%)
ADP- [ U- $^{14}\text{C}$ ] glucose	2.22	$1.49 \pm 0.03$	67
UDP- [ U- $^{14}\text{C}$ ] glucose	2.22	$1.58 \pm 0.02$	71
[ U- $^{14}\text{C}$ ] G1P	2.22	$1.66 \pm 0.03$	75

\* mean of triplicate analyses  $\pm$  SD

Table 5.2 Retention and elution efficiencies

radiochemical standards (Amersham)	unswirled (cpm $\times 10^{-4}$ )	swirled (cpm $\times 10^{-4}$ )*	retention (%)	elution (%)
ADP-[ U- $^{14}\text{C}$ ] glucose	7.25	$5.51 \pm 0.01$	76	24
UDP-[ U- $^{14}\text{C}$ ] glucose	7.06	$5.88 \pm 0.01$	83	17
[ U- $^{14}\text{C}$ ] G1P	6.93	$0.30 \pm 0.001$	4	96

\* mean of triplicate analyses  $\pm$  SD

Table 5.3 Equations for the conversion of cpm to units of enzyme activity

(1) ADP-glucose pyrophosphorylase activity (n kat per endosperm)

$$\text{activity} = \frac{\text{cpm} \times 1.964 \times 6.126 \times 10^{-4} \times A \times B \times 1.07}{C \times 10^{-3} \times D \times E} \quad \text{n kat per endosperm}$$

(2) UDP-glucose pyrophosphorylase activity (n kat per endosperm)

$$\text{activity} = \frac{\text{cpm} \times 1.697 \times 6.126 \times 10^{-4} \times A \times B \times 1.07}{C \times 10^{-3} \times D \times E} \quad \text{n kat per endosperm}$$

(3) ADP-glucose pyrophosphorylase initial reaction velocities ( $v_o$ ) (n kat per ml of enzyme extract)

$$v_o = \frac{\text{cpm} \times 1.964 \times 6.126 \times 10^{-4} \times 1.07}{C \times 10^{-3} \times D} \quad \text{n kat per ml of enzyme extract}$$

where A = dilution factor for the endosperm extract

B = volume of extract (ml)

C = extract aliquot ( $\mu$ l)

D = assay digest time (s)

E = no. of endosperms

\* 1 dpm =  $6.126 \times 10^{-4}$  n moles of  $[U-^{14}C]$  nucleoside diphosphate sugar.

+ dilution factor caused by the addition of alkaline phosphatase = 1.07

In order to determine whether the products synthesised were indeed nucleoside diphosphate sugars the reaction products were chromatographed as described in methods section 5.2.5. Examples of the data obtained are shown in tables 5.4 and 5.5. The pyridine/acetic acid buffer at pH 6.5 gave the best separation of the four solvent systems that were investigated, although the results for the other systems were qualitatively similar. Analyses of the lanes by strip scintillation counting showed that the peaks of radioactivity coincided with the spots visualised by chemical staining. In each solvent system the radioactive product co-chromatographed with the radiochemical standard (results not shown).

Table 5.4 Product identification

Paper electrophoresis in pyridine/acetic acid buffer at pH 6.5 for  
55 min at 3 000 volts

Sample	phosphate stain		scintillation counting	
	distance travelled (cm)	mobility relative to $[U-^{14}C]G1P$ $R_{G1P}$	distance travelled (cm)	mobility relative to $[U-^{14}C]G1P$ $R_{G1P}$
$[U-^{14}C]G1P$	27.5	1.00	26.8	1.00
ADP- $[U-^{14}C]$ glucose	23.3	0.85	22.8	0.85
UDP- $[U-^{14}C]$ glucose	30.0	1.09	29.9	1.12
*endosperm (40% cut)	23.1	0.84	22.8	0.85
*leaf (50% cut)	22.9	0.83	22.8	0.85
*leaf+3-PGA (50% cut)	23.5	0.85	22.8	0.85

\* ATP-dependent reaction products

Table 5.5 Product identification

Descending paper chromatography in ethylacetate -pyridine - H<sub>2</sub>O (8:2:1) for 48h

Sample	p-anisidine stain		scintillation counting
	distance travelled (cm)	mobility relative to glucose (R <sub>g</sub> )	distance travelled (cm)
glucose	25.6	1.00	* N/A
ADP-glucose hydrolysate	24.1	0.97	24.6
UDP-glucose hydrolysate	24.5	0.96	25.6

\* [ U-<sup>14</sup>C ] glucose was not available for use as a mobility standard.

### 5.3.2 ADP- and UDP-glucose pyrophosphorylase activities during endosperm development

The activities of ADP- and UDP-glucose pyrophosphorylases in developing endosperms are shown in fig 5.1. The data are expressed in n kat per endosperm. The results for the extractions at 50 days after anthesis derive from whole caryopses minus their embryos. These caryopses were ground for 5 s in a Moulinex Type 299.200 Brevete grinder prior to homogenisation as described in section 5.2.3. This procedure was necessary for satisfactory extraction.

The respective developmental patterns for the 2 enzymes were different. ADP-glucose pyrophosphorylase activity (fig 5.1a) rose quite sharply from a minimum (barely detectable) at 13 days after

Figure 5.1 The activities of ADP- and UDP- glucose pyrophosphorylases during endosperm development

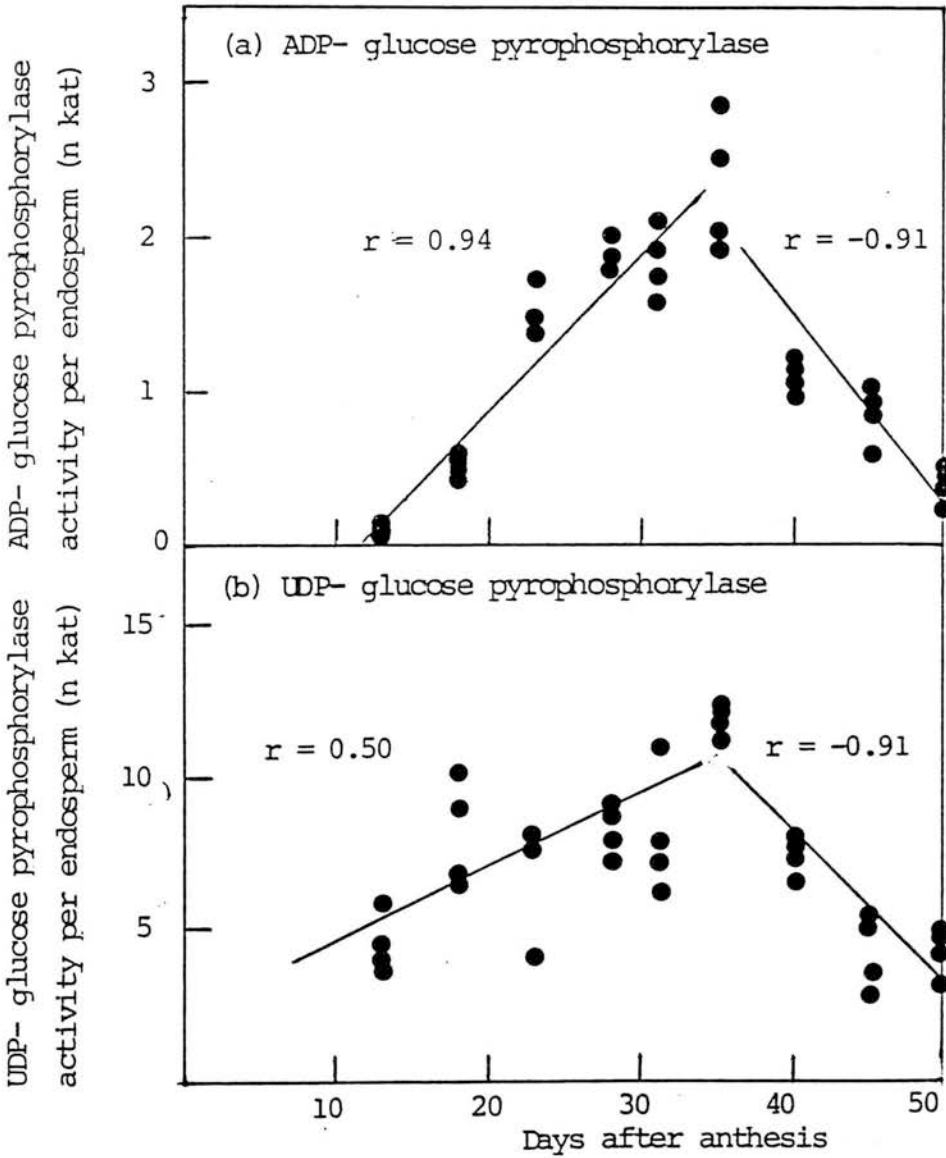


Figure 5.2 Ratio of UDP-glucose pyrophosphorylase activity  
to ADP- glucose pyrophosphorylase activity in  
developing endosperms

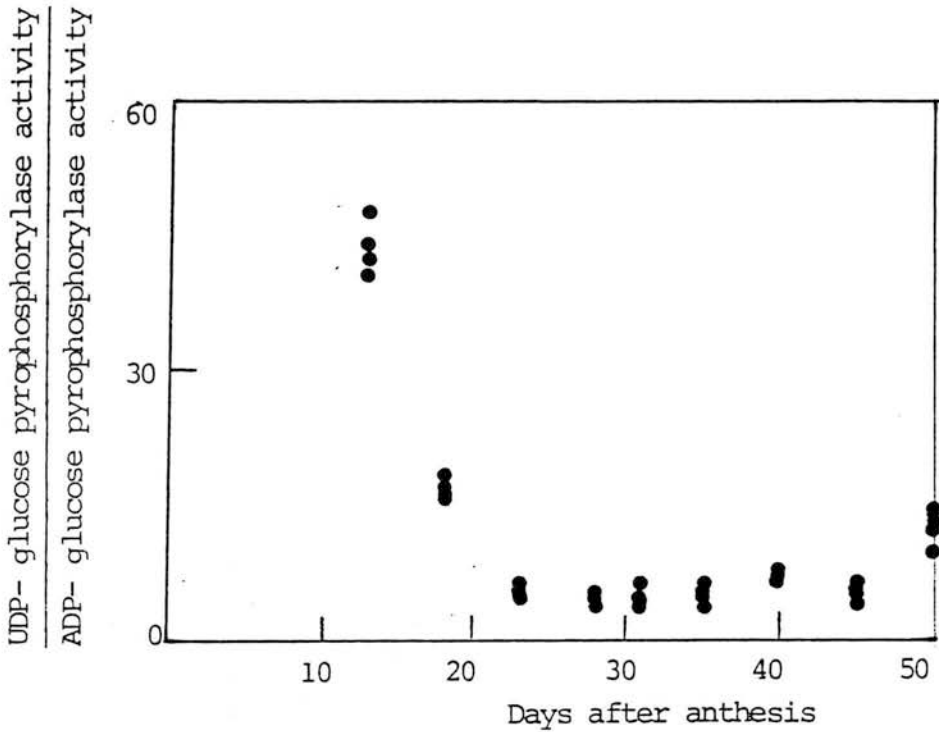
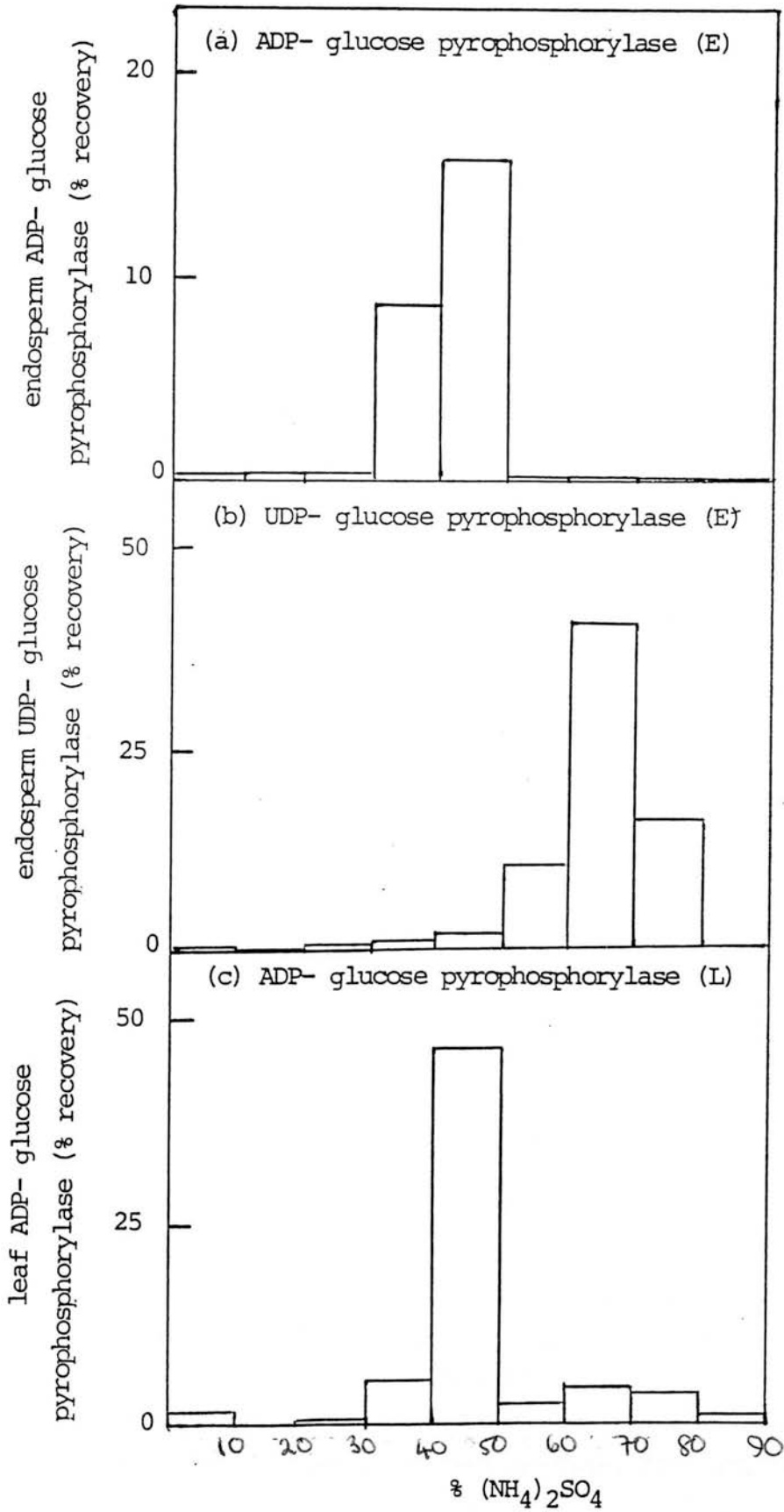


Figure 5.3  $(\text{NH}_4)_2\text{SO}_4$  fractionation, percentage recovered activity

( Key: E = endosperm, L = leaf )





anthesis to a maximum at 35 days after anthesis. This rise in activity was characterised by a high positive correlation with 89 per cent of the data having a linear relationship with time.

The enzyme activity at the maximum was approximately 23 times that measured in the early stages of endosperm development. This rapid rise to the maximum level of activity was followed by a sharp decline which was essentially linear with a high negative correlation ( $r = -0.91$ ). The final levels of activity were approximately 7 times less than those measured at 35 days after anthesis.

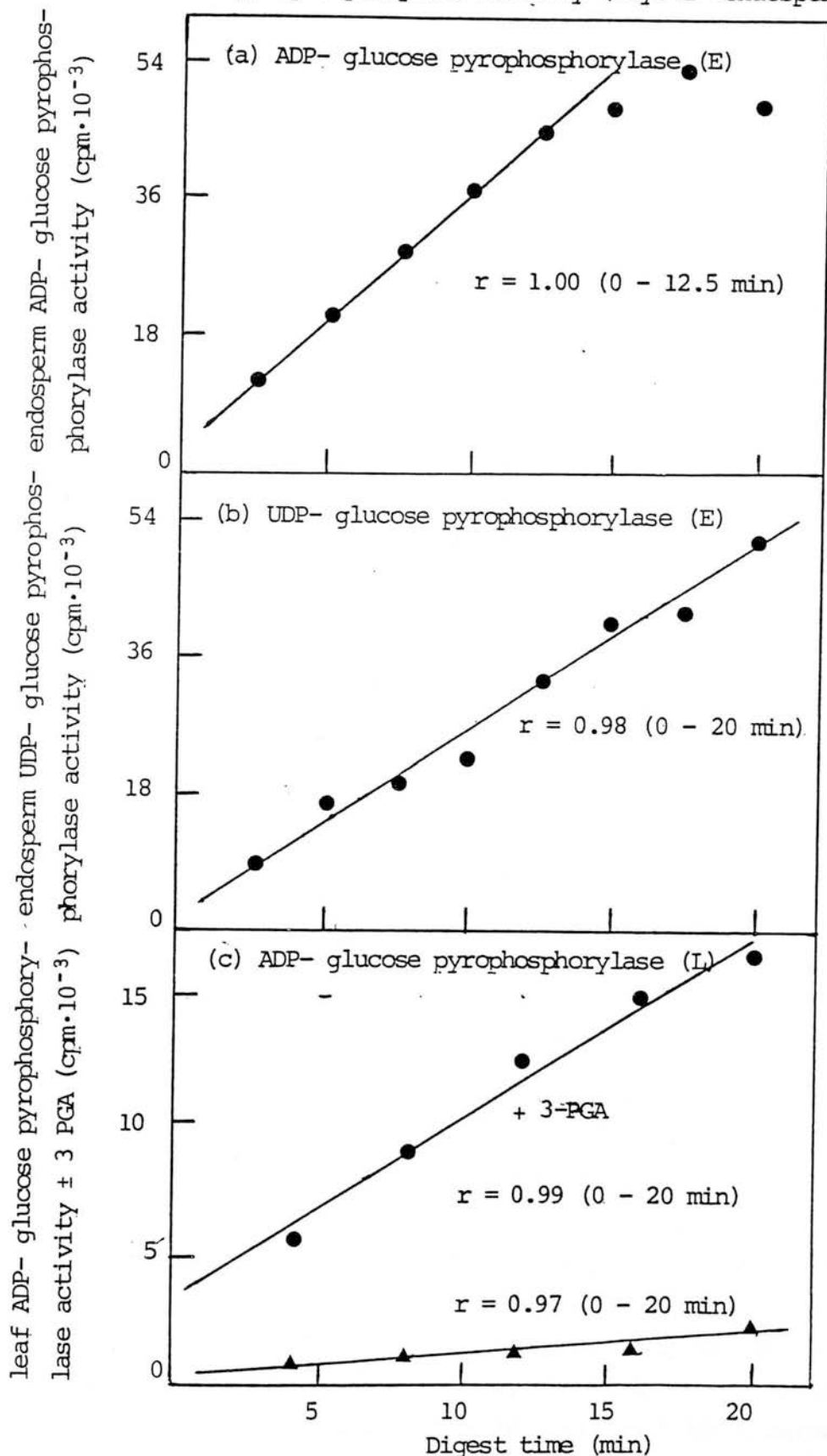
UDP-glucose pyrophosphorylase activity (fig 5.1b) in the developing endosperms rose slowly from a relatively high level of activity (40-45 times higher than ADP-glucose pyrophosphorylase fig 5.2) to a slightly increased activity by 35 days after anthesis. The slow rate of increase had a positive correlation but only 64 per cent of the values had a linear relationship with time. The activity then declined at a similar rate to that observed for ADP-glucose pyrophosphorylase and the final levels were approximately 12 times higher than those for the ATP-dependent activity (fig 5.2).

### 5.3.3 Ammonium sulphate fractionation

Examples of the percentage recoveries of ADP- and UDP-glucose pyrophosphorylase activities following ammonium sulphate fractionation of endosperm and leaf preparations are shown in fig 5.3.

Active endosperm ADP-glucose pyrophosphorylase (fig 5.3a) was recovered mainly in the 40 and 50 per cent fractions while the precipitation of endosperm UDP-glucose pyrophosphorylase (fig 5.3.b) required higher concentrations of ammonium sulphate. Although the latter activity was found predominately in the 70 per cent fraction, there was considerable detectable activity in the 40 and 50 per cent ammonium sulphate fractions. The total recovery of

Figure 5.4 Linearity of endosperm ATP- and UTP- dependent 128.  
 pyrophosphorylase activity and ATP- dependent leaf  
 pyrophosphorylase activity (Key: E =endosperm, L =leaf )



active endosperm ADP-glucose pyrophosphorylase was 26 per cent of that present in the unfractionated preparation. In contrast, 85 per cent of the initial UDP-glucose pyrophosphorylase activity was recovered by ammonium sulphate precipitation.

Ammonium sulphate fractionation of leaf ADP-glucose pyrophosphorylase (fig 5.3c) recovered 67 per cent of that present in the crude extract and the majority of this activity was precipitated in the 50 per cent fraction.

Unless otherwise stated, the remaining studies reported in this section relate to enzyme activities as precipitated by ammonium sulphate. The 40 per cent endosperm fraction was used for studies on both ADP- and UDP-glucose pyrophosphorylases, while the 50 per cent leaf fraction was utilised for work on leaf ADP-glucose pyrophosphorylase.

#### 5.3.4 Linearity of the radiochemical assay for ADP- and UDP-glucose pyrophosphorylases

Fig 5.4 shows the activities of both endosperm (ATP- and UTP-dependent) and leaf (ATP-dependent) activities under the conditions of the radiochemical assay. The results are plotted as raw data in cpm as a function of digest time (min). Leaf ATP-dependent activity is shown in the presence and absence of 3-PGA (1mM).

Endosperm ATP-dependent activity (fig 5.4a) was linear for a minimum of 10 min, reaching a count of  $45 \times 10^3$  cpm before beginning to level off while UTP-dependent activity (fig 5.4b) had a slightly slower response rate remaining linear for 20 min. For all future assays, the activities of both ADP-glucose and UDP-glucose pyrophosphorylases were suitably diluted to ensure that the maximum incorporation of radio-label did not generally exceed a measured count of  $45 \times 10^3$  cpm (the maximum possible count, allowing for quenching, was approximately  $77 \times 10^3$  cpm). Leaf ADP-glucose

Figure 5.5 pH- dependent activities of endosperm (E) ADP- and UDP- glucose pyrophosphorylases and leaf (L) ADP- glucose pyrophosphorylase <sup>130.</sup>

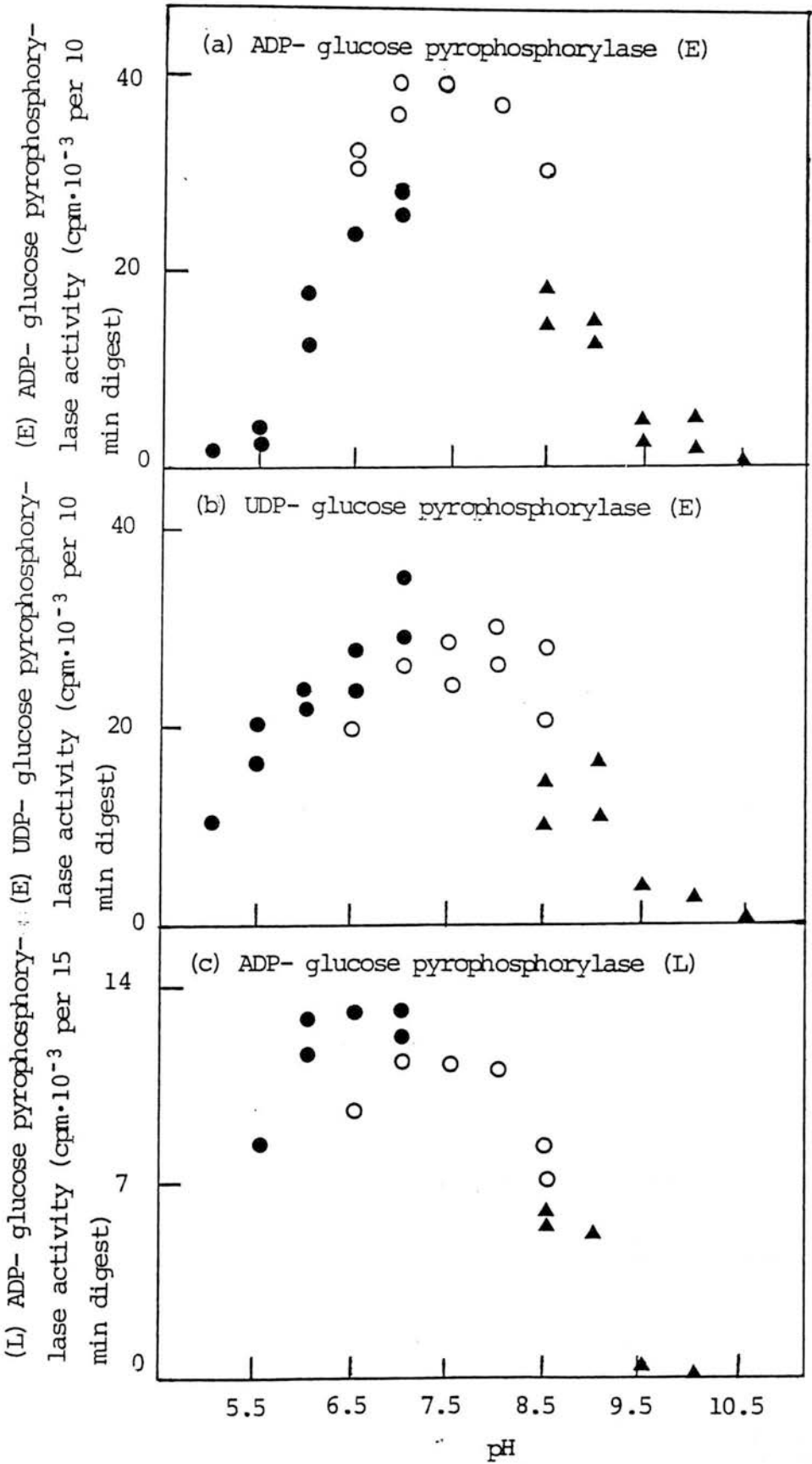
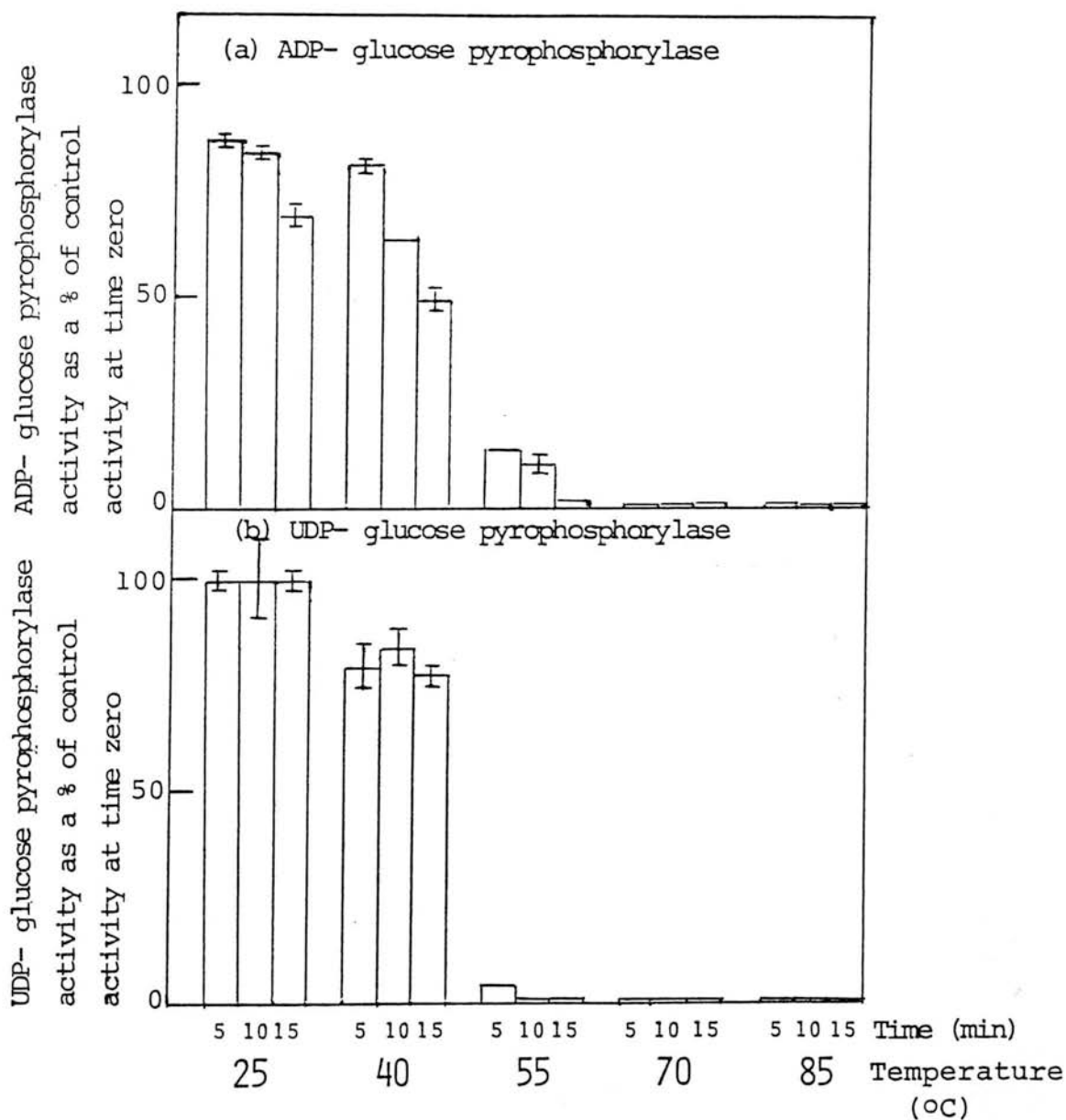


Figure 5.6 Thermal characteristics of endosperm ADP- and UDP-glucose pyrophosphorylases with respect to pre-assay incubation time and temperature



pyrophosphorylase (fig 5.4c) responded very slowly in the absence of 3PGA (1mM) and considerably faster in the presence of 3-PGA (1mM). Both activities were linear under the conditions of the assay.

#### 5.3.5 pH-dependent activity

The pH profiles of endosperm ADP- and UDP-glucose pyrophosphorylases together with leaf ADP-glucose pyrophosphorylase are shown in fig 5.5. The results are expressed in cpm per digest time (min). Where the results from duplicate analyses did not differ, a single datum point is shown.

Endosperm ADP-glucose pyrophosphorylase (fig 5.5a) showed a bell shaped activity curve with a maximum between pH 7.0 and 8.0. Endosperm UDP-glucose pyrophosphorylase (fig 5.5b) showed maximum activity between pH 6.5 and 8.5. Considerable activity was detected at pH 5.0, approximating to 35 per cent of the maximum.

Leaf ADP-glucose pyrophosphorylase (fig 5.5c) had a broad maximum between pH 6.0 and 8.0. No data was obtained below pH 5.5

#### 5.3.6 Thermal characteristics of endosperm ADP- and UDP-glucose pyrophosphorylases

The response to temperature of endosperm ADP- and UDP-glucose pyrophosphorylases is shown in fig 5.6. The results are expressed as a percentage of the activity assayed under the standard assay conditions (section 5.2.4.1/3). The bars in the histograms represent the range of the duplicate analyses.

ATP-dependent activity (fig 5.6a) declined both as a function of incubation time and temperature. This observation was apparent even at 25°C when, following a pre-assay incubation time of 15 min at this temperature, the activity was reduced to 70 per cent of the control. The activity dropped quite sharply at 55°C and was

Figure 5.7 Gel filtration (Ultrogel AcA 34) chromatography of endosperm ADP- glucose pyrophosphorylase at pH 8.0 in HEPES (20mM) containing mercaptoethanol (20mM)

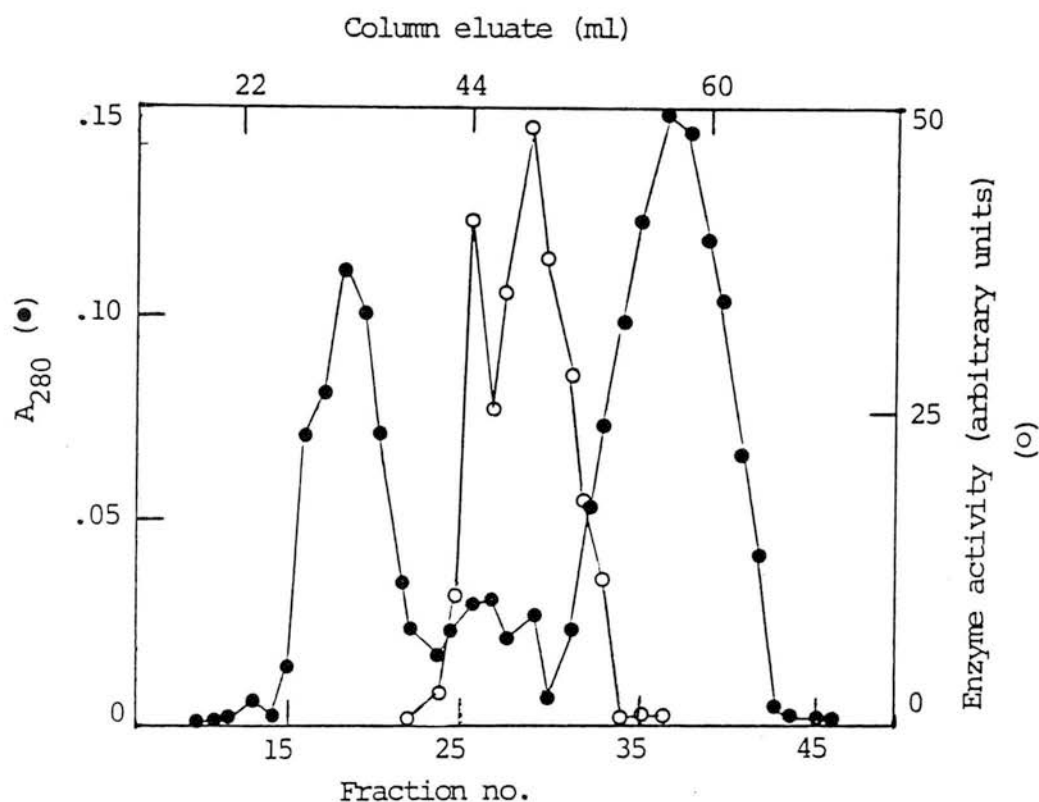
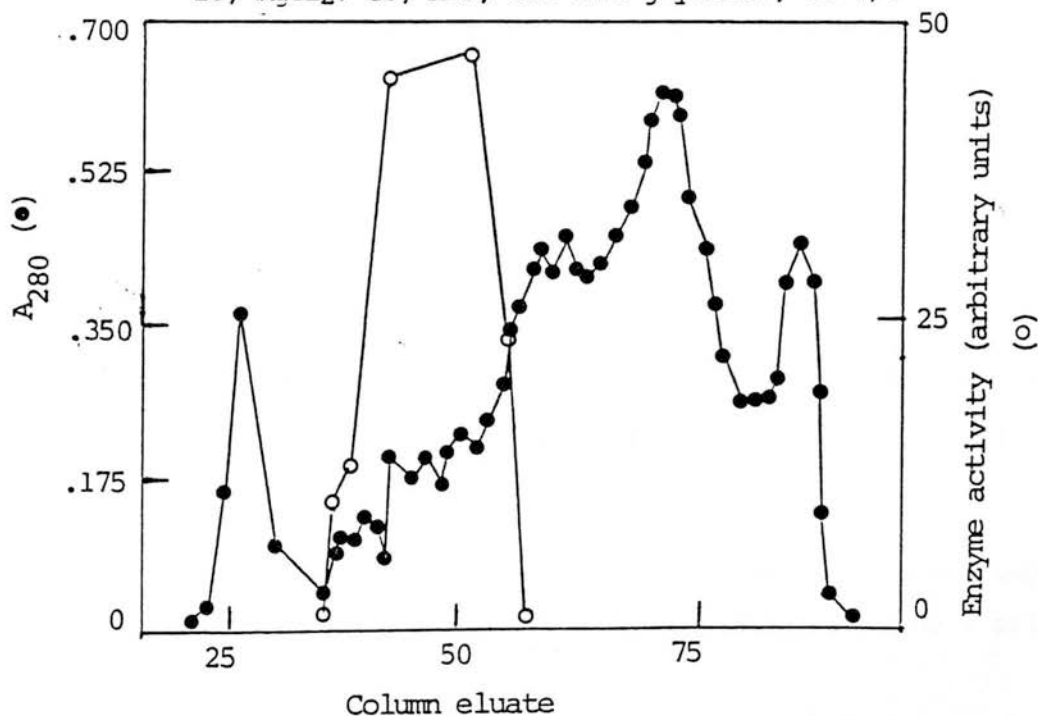


Figure 5.8 Gel filtration (Ultrogel AcA 34) chromatography of endosperm ADP- glucose pyrophosphorylase at pH 8.0 in triethanolamine (20mM) containing (mM): mercaptoethanol, 20;  $MgCl_2$ , 10; ATP, 0.2 and glycerol, 2% w/v



inactivated at higher temperatures.

UTP-dependent activity (fig 5.6b) was stable at 25°C and retained the activity of the control digest. Activity declined to approximately 80 per cent of the control at 40°C and thereafter was rapidly inactivated at higher temperatures.

### 5.3.7 Gel filtration and ion exchange chromatography

Gel filtration was utilised to purify further endosperm ADP-glucose pyrophosphorylase which had been precipitated from the crude extracts by ammonium sulphate fractionation. Active enzyme protein, precipitated in broad ammonium sulphate cuts (30 - 60%), was initially chromatographed at pH 8.0 in HEPES buffer and a typical example of this procedure is shown in fig 5.7. Enzyme activity is given in arbitrary units and relates to the assay procedures described in section 5.2.4.2. The active fractions were eluted between the totally excluded and totally included proteins, however subsequent assay of the pooled active fractions by the radiochemical assay (section 5.2.4.1) did not detect any ATP-dependent activity.

The buffer system was changed to incorporate essentially the components of the homogenisation buffer (section 5.2.3) and samples of the same extract were applied to the column as described in section 5.2.9. Fig 5.8 shows the essential parameters as defined by this system. As with the previous buffer system, active protein was eluted within the effective fractionation range of the Aca34 permeation gel. Subsequent analyses of the pooled fractions (following concentration by 60 per cent ammonium sulphate, section 5.2.9) either by the radiochemical assay (section 5.2.4.1) or by the ADP-glucose dependent assay (section 5.2.4.2) showed a complete loss of enzyme activity.

Samples of the narrow (40 per cent) ammonium sulphate cuts were applied to a DEAE-cellulose column and the protein eluted by a salt



Figure 5.9 DEAE- cellulose chromatography of ATP- and UTP- dependent endosperm pyrophosphorylase activity at pH 8.0 in triethanolamine (20mM) containing (mM) : DTE, 2;  $MgCl_2$ , 10; ATP, 0.2; and glycerol, 2% w/v. 135.

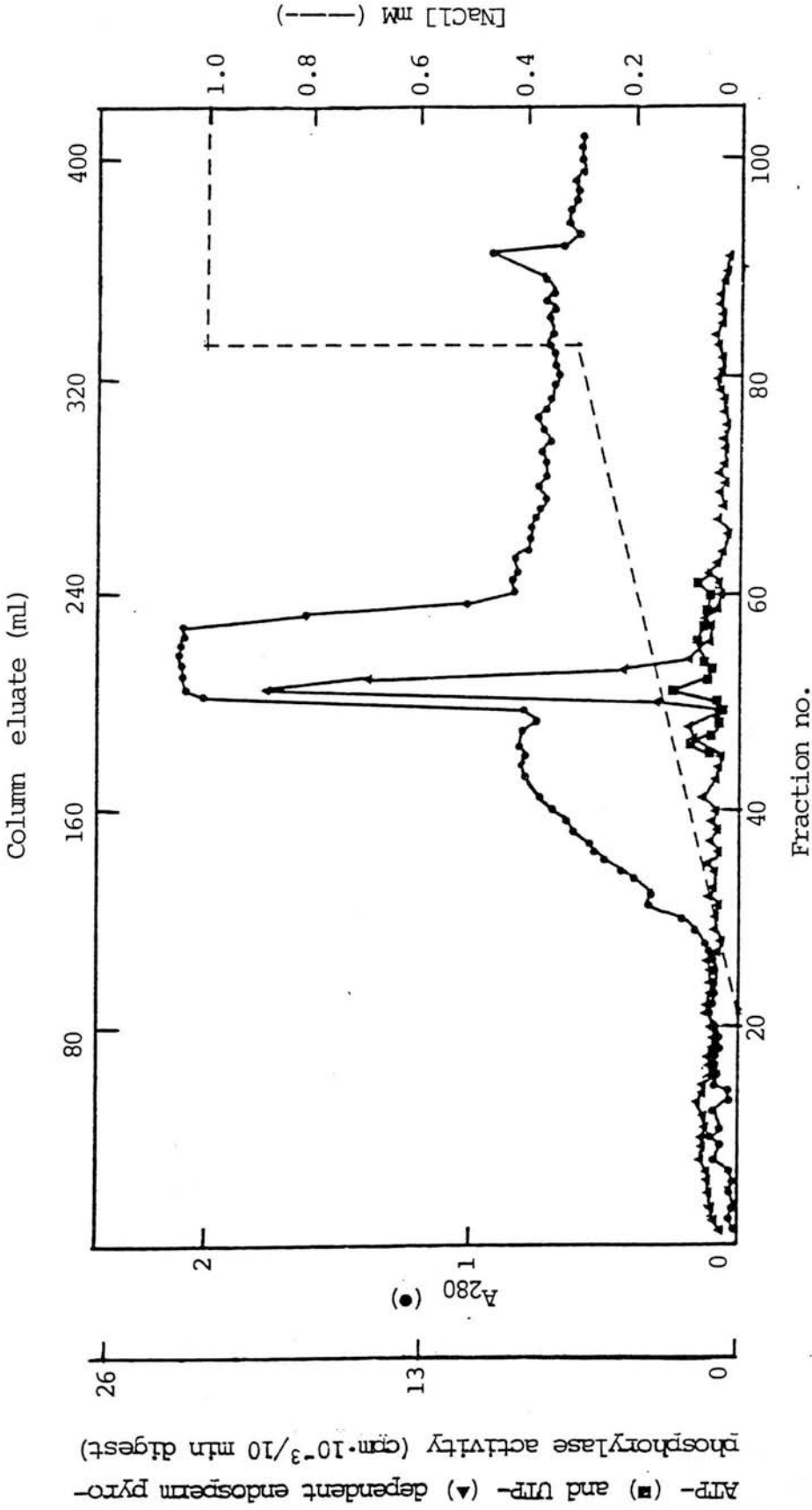
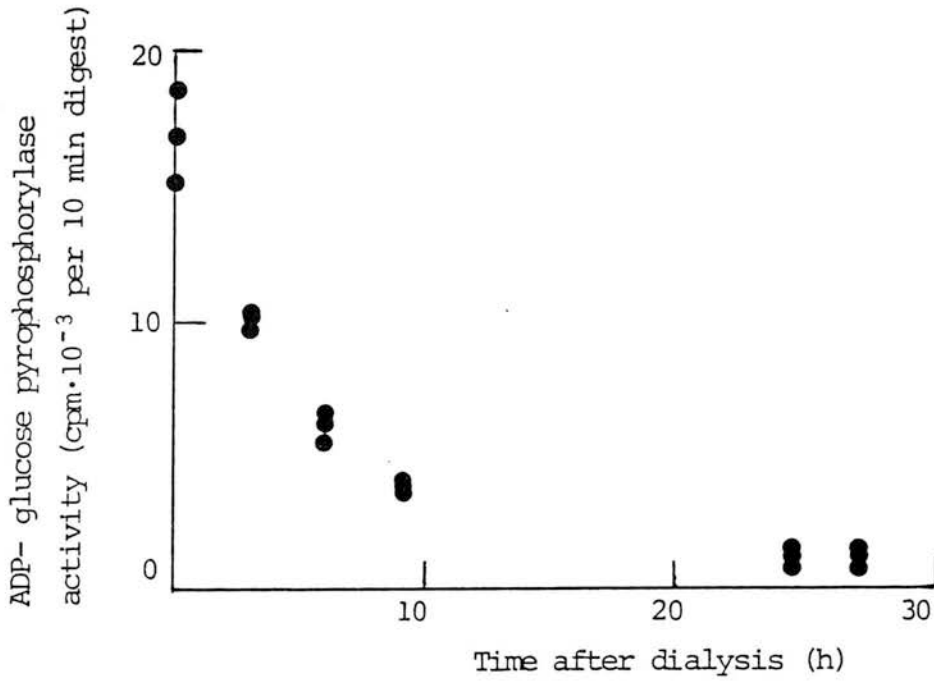


Figure 5.10 Activity of endosperm ADP- glucose pyrophosphorylase as a function of time after dialysis



gradient. All fractions were analysed for ATP- and UTP-dependent pyrophosphorylase activity by the radiochemical assay. The analyses were carried out as the fractions were eluted. An example of this procedure is given in fig 5.9. A very sharp peak of UTP-dependent activity was detected at fraction 51 when the salt concentration was 150mM. There was no significant ATP-dependent activity detected throughout the run (35h).

#### 5.3.8 Time-dependent activity of ADP-glucose pyrophosphorylase

Evidence from the temperature/time experiments (section 5.3.6) suggested that following dialysis of the 40 per cent ammonium sulphate fraction, the activity of ADP-glucose pyrophosphorylase declined with respect to time.

Fig 5.10 shows the results from an experiment designed to investigate this phenomenon. The data show a significant decline in activity within 3h after dialysis with the preparation losing approximately half its ATP-dependent activity at 4h after dialysis. Subsequent experiments were designed to minimise this loss in activity with the maximum number of digests being restricted to 30 per experimental set. No such restrictions were necessary for the assay of either endosperm UDP-glucose pyrophosphorylase or leaf ADP-glucose pyrophosphorylase which were both stable after dialysis.

#### 5.3.9 Metabolite activation or inhibition of endosperm and leaf ADP-glucose pyrophosphorylases

Various metabolites were screened for possible activation or inhibition effects on ADP-glucose pyrophosphorylase activities from endosperm and leaf tissues. For reasons explained in the previous section, the metabolites were tested in blocks of 13 in the presence and absence of ATP. This resulted in 26 digests per block, a total of 152 digests. The results of this screen are given in table 5.6.

Table 5.6 Metabolite activation/inhibition of endosperm and leaf  
ADP-glucose pyrophosphorylase

Metabolite (1mM)	Endosperm tissue		Leaf tissue	
	activity (cpm/10 min digest)	activation (fold)	activity (cpm/15 min digest)	activation (fold)
control	52 264	1.0	3 089	1.0
3-PGA	50 519	1.0	19 738	6.4
2-PGA	44 700	0.9	16 499	5.3
GAP	44 864	0.9	2 719	0.9
2-deoxy.glc.	47 673	0.9	3 878	1.3
pyruvate	37 026	0.7	2 666	0.9
PEP	39 747	0.8	7 011	2.3
F-2, 6-bisP	43 314	0.8	3 221	1.0
F-1, 6-bisP	42 849	0.8	6 004	1.9
G-1, 6-bisP	31 486	0.6	3 407	1.1
Malic acid	37 679	0.7	2 761	0.9
G6P	36 945	0.7	7 046	2.3
DHAP	37 272	0.7	* NM	NM
control	30 664	1.0	2 509	1.0
lactic acid	35 252	1.1	2 612	1.0
glutamine	26 957	0.9	2 019	0.8
sucrose	34 342	1.1	1 259	0.5
glutamate	29 082	0.9	1 655	0.7
AMP	30 064	1.0	1 229	0.5
EDTA	19 047	0.6	2 169	0.9
β G1P	33 105	1.1	2 536	1.0
UDP	44 056	+ N/A	35 400	N/A
ADP	16 596	0.5	1 454	0.6
α KG	33 486	1.1	2 363	0.9
citrate	27 937	0.9	1 960	0.8
3-PGA	29 540	1.0	15 852	6.3
control	26 573	1.0	1 900	1.0
3-PGA	24 823	0.9	17 032	9.0
CDP	22 985	0.9	1 803	0.9
ADP-glucose	14 417	0.5	1 598	0.8
UDP-glucose	24 285	0.9	2 736	1.4
PPi	18 670	0.7	394	0.2
Pi	22 055	0.8	962	0.5
F6P	28 146	1.1	3 990	2.1
NADP	23 690	0.9	2 868	1.5
GDP	22 546	0.8	1 717	0.9
ascorbate	23 717	0.9	1 993	1.0
phytic acid	26 165	1.0	1 479	0.8
DHAP	-	-	3 246	1.7

\* NM = not measured

+ N/A = not applicable, see text.

There was no significant activation of the endosperm enzyme with any of the metabolites. The apparent increase in activity in the presence of UDP was assigned to UTP-dependent activity brought about by the conversion of UDP to UTP by nucleoside diphosphate kinase activity. This enzyme was shown later (table 5.8) to be a contaminant of both the endosperm and the leaf preparations. Considerable inhibition of endosperm ADP-glucose pyrophosphorylase was evident in the presence of G-1, 6-bisP, EDTA, ADP, ADP-glucose and PPI.

The leaf enzyme was activated up to 9 times by 3-PGA, 5 times by 2-PGA and to lesser extents by PEP, F-1, 6-bisP, G6P, F6P and DHAP. The presence of UDP in the digest had a similar effect to that observed for the endosperm enzyme. PPI and Pi both inhibited the leaf enzyme.

Time did not permit proper replication of the experimental data shown in table 5.6, however the major observations were incorporated into later experiments (section 5.3.12).

#### 5.3.10 Determinations of $K_m$ for endosperm and leaf ADP-glucose pyrophosphorylases initial reaction velocities

The G1P- and ATP-dependent pyrophosphorylase initial reaction velocities were investigated for both the endosperm and leaf enzyme preparations. The apparent  $K_m$  values were calculated by the procedures previously discussed in section 4.4.2.1. The initial reaction velocities shown in cpm per 10 min digest and cpm per 15 min digest for the endosperm and leaf enzymes respectively. These initial reaction velocities together with the estimations for calculated maximum velocities can be converted to enzyme units (n kat per ml of enzyme extract) by the equations detailed in table 5.3. The apparent maximum velocities have been expressed in this manner.

Figure 5.11 G1P- dependent endosperm ADP- glucose pyrophosphorylase  
initial reaction velocities ( 0 - 1.40 mM G1P)

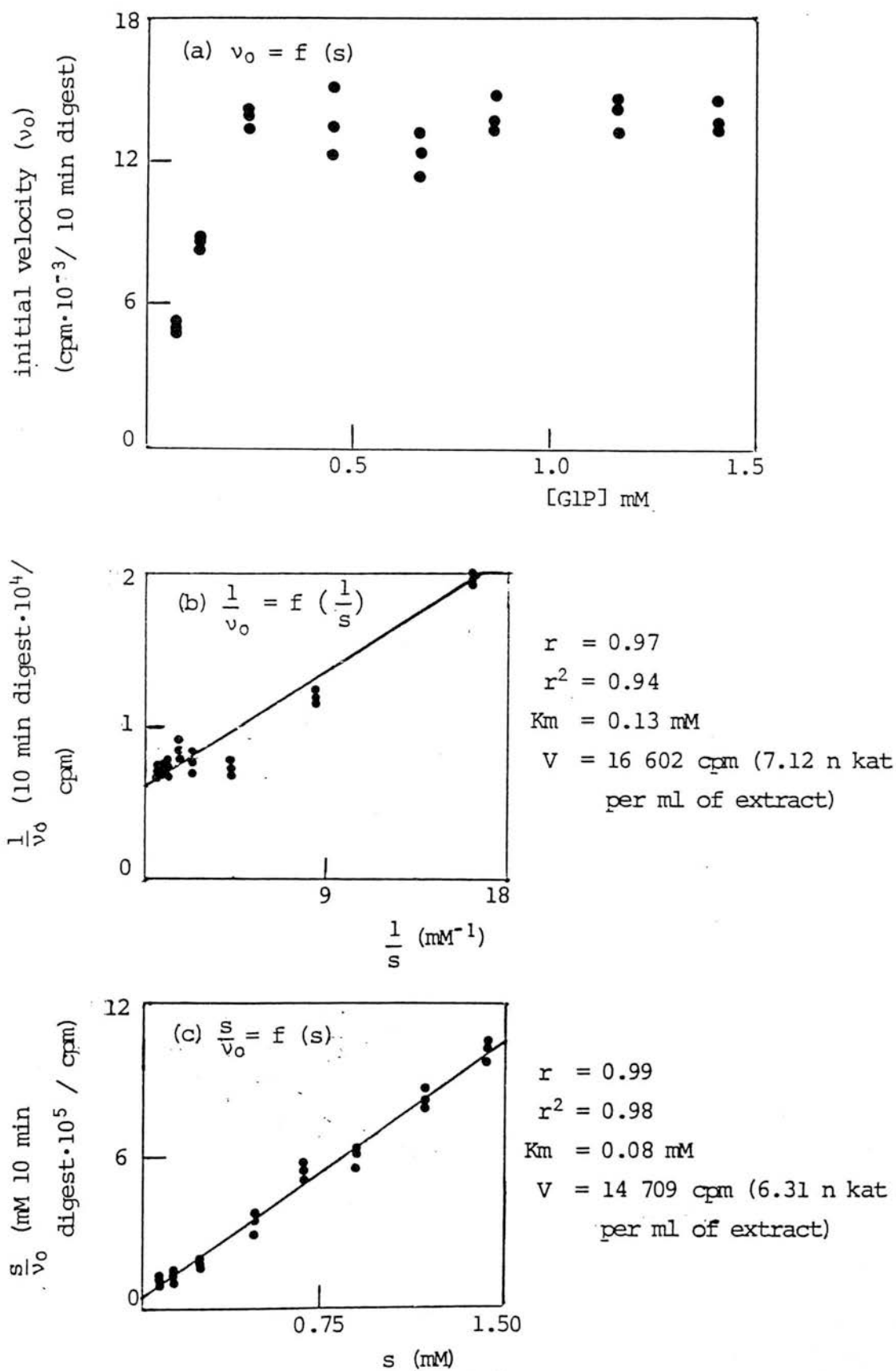


Figure 5.12 GIP- dependent endosperm ADP- glucose pyrophosphorylase  
initial reaction velocities ( 0 - 0.28 mM GIP )

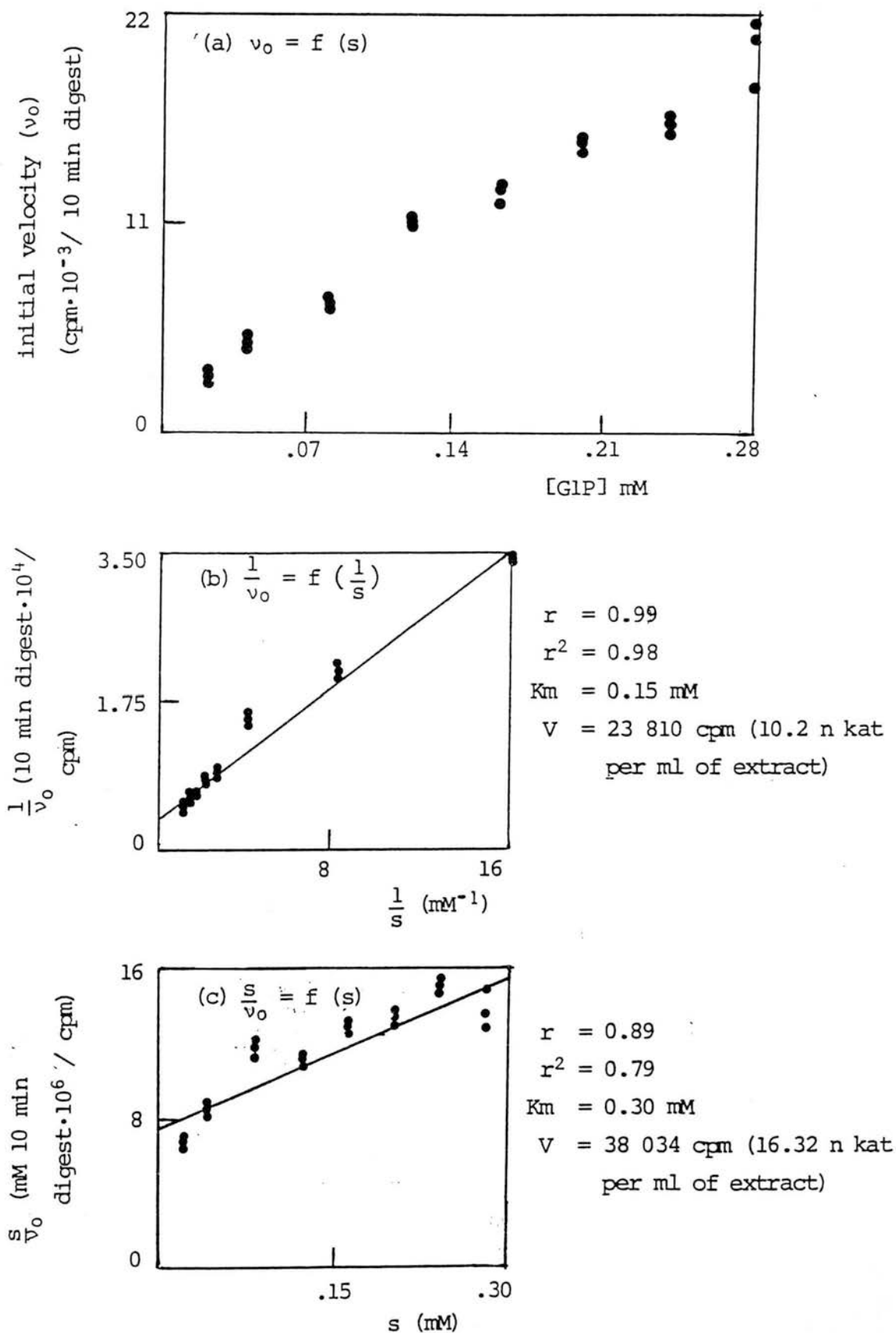
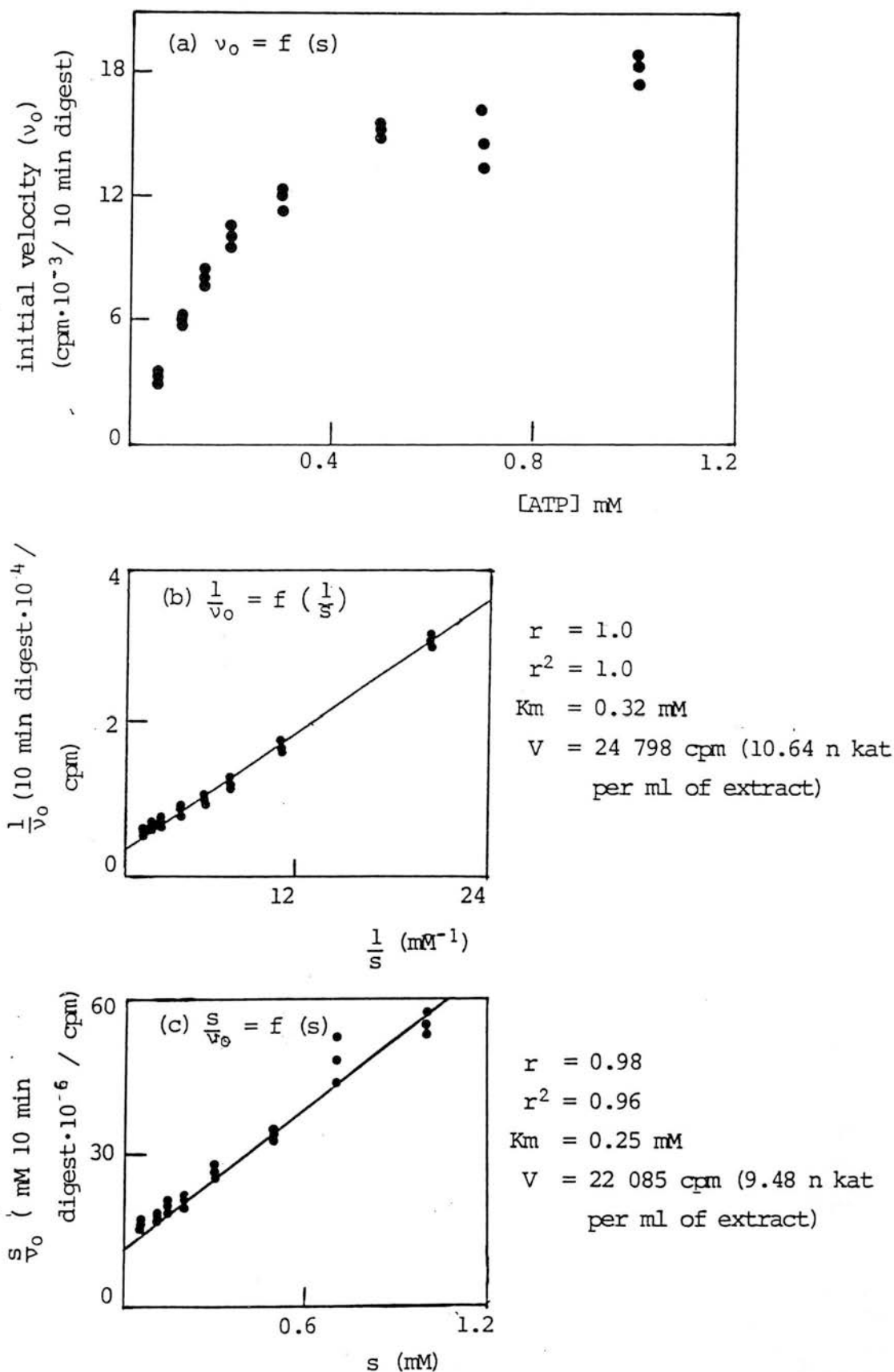


Figure 5.13 ATP- dependent endosperm ADP- glucose pyrophosphorylase  
initial reaction velocities ( 0 - 1.0 mM ATP )





G1P- dependent endosperm ADP-glucose pyrophosphorylase initial reaction velocities were determined over 2 concentration ranges. Fig 5.11 shows the results obtained from varying G1P in the enzyme digest from 0 - 1.4mM. Apparent maximum velocity was attained at a G1P concentration of 0.25mM (fig 5.11a) and the double reciprocal plot of this data (fig 5.11b) gave a calculated  $K_m$  (G1P) of 0.13mM. The subsequent single reciprocal plot (fig 5.11c) produced a lower calculated  $K_m$  of 0.08mM. In a second experiment, carried out over a narrower concentration range of between 0 and 0.28 mM G1P (fig 5.12a), the calculated  $K_m$  (G1P) values were higher than those obtained previously (fig 5.11). The double reciprocal plot (fig 5.12b) showed an apparent  $K_m$  (G1P) value of 0.15mM while the single reciprocal plot (fig 5.12c) gave a value of 0.30mM. The raw data (fig 5.12a) obtained at a G1P concentration of 0.28mM show 2 values which may be erroneous. These values are clearly seen on the single reciprocal plot (fig 5.12c). If they are excluded from the calculation of  $K_m$  (G1P) in fig 5.12c, then the apparent  $K_m$  (G1P) re-computes to 0.25mM (data not shown).

ATP-dependent endosperm ADP-glucose pyrophosphorylase initial reaction velocities were determined by varying the concentration of ATP in the enzyme digest from 0 - 1.0mM (fig 5.13). The graph of initial velocity versus substrate concentration (fig 5.13a) showed a hyperbolic curve which, when replotted by both procedures (figs 5.13b and 5.13c) gave apparent  $K_m$  (ATP) values of 0.32 and 0.25 mM respectively.

The apparent  $K_m$  values (G1P and ATP) for leaf ADP-glucose pyrophosphorylase were calculated in a similar manner except that 3-PGA (1 mM) was included in the assay digest. In the absence of 3-PGA the initial reaction velocities of the leaf enzyme were extremely low (table 5.7) and the apparent kinetic constants were not calculated.

Figure 5.14 GLP- dependent leaf ADP- glucose pyrophosphorylase  
initial reaction velocities + 1mM 3 PGA ( 0 -0.56 mM GLP)

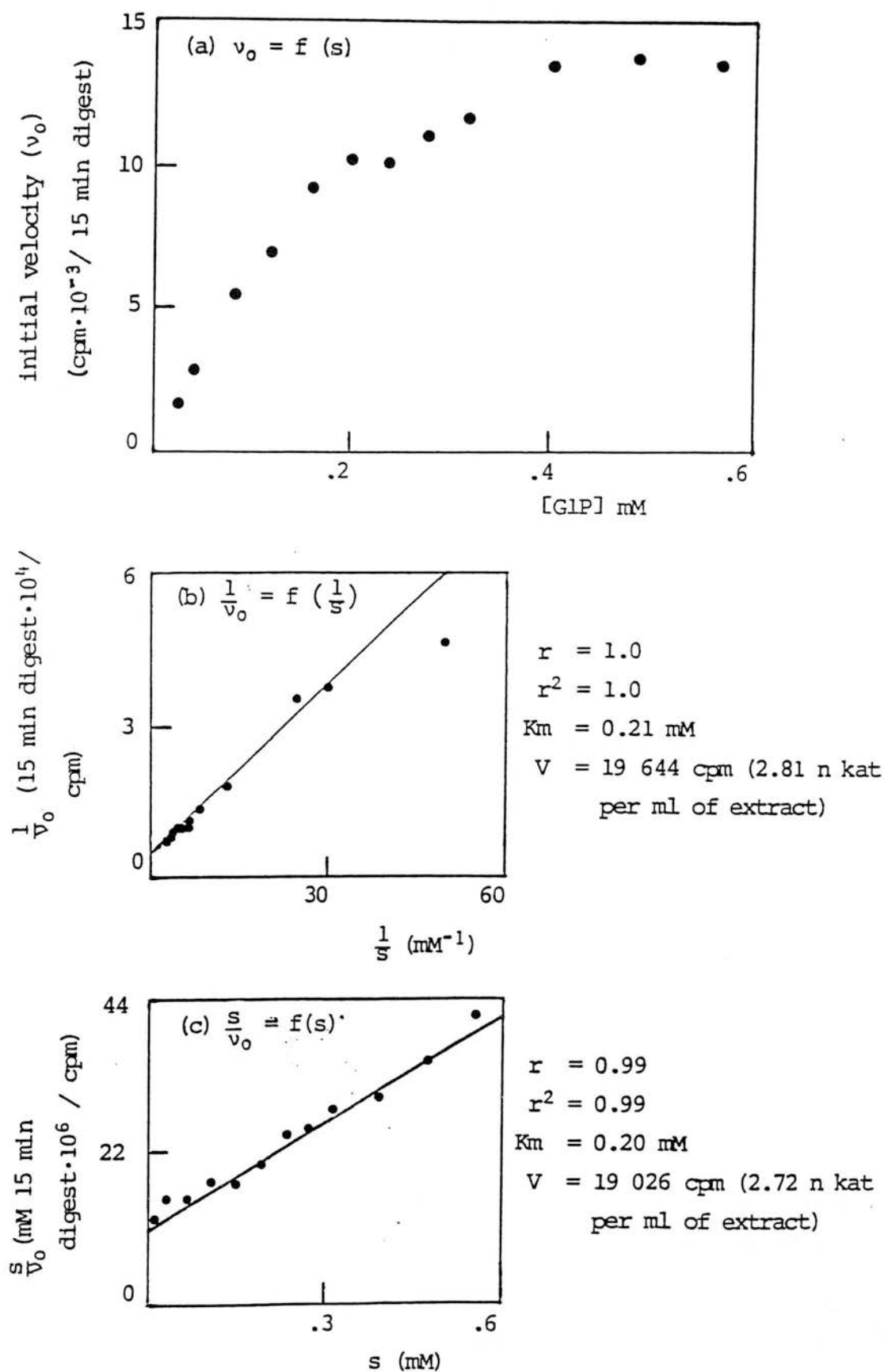


Figure 5.15 ATP- dependent leaf ADP- glucose pyrophosphorylase 145.  
initial reaction velocities + 3 PGA ( 0 - 1.0 mM ATP )

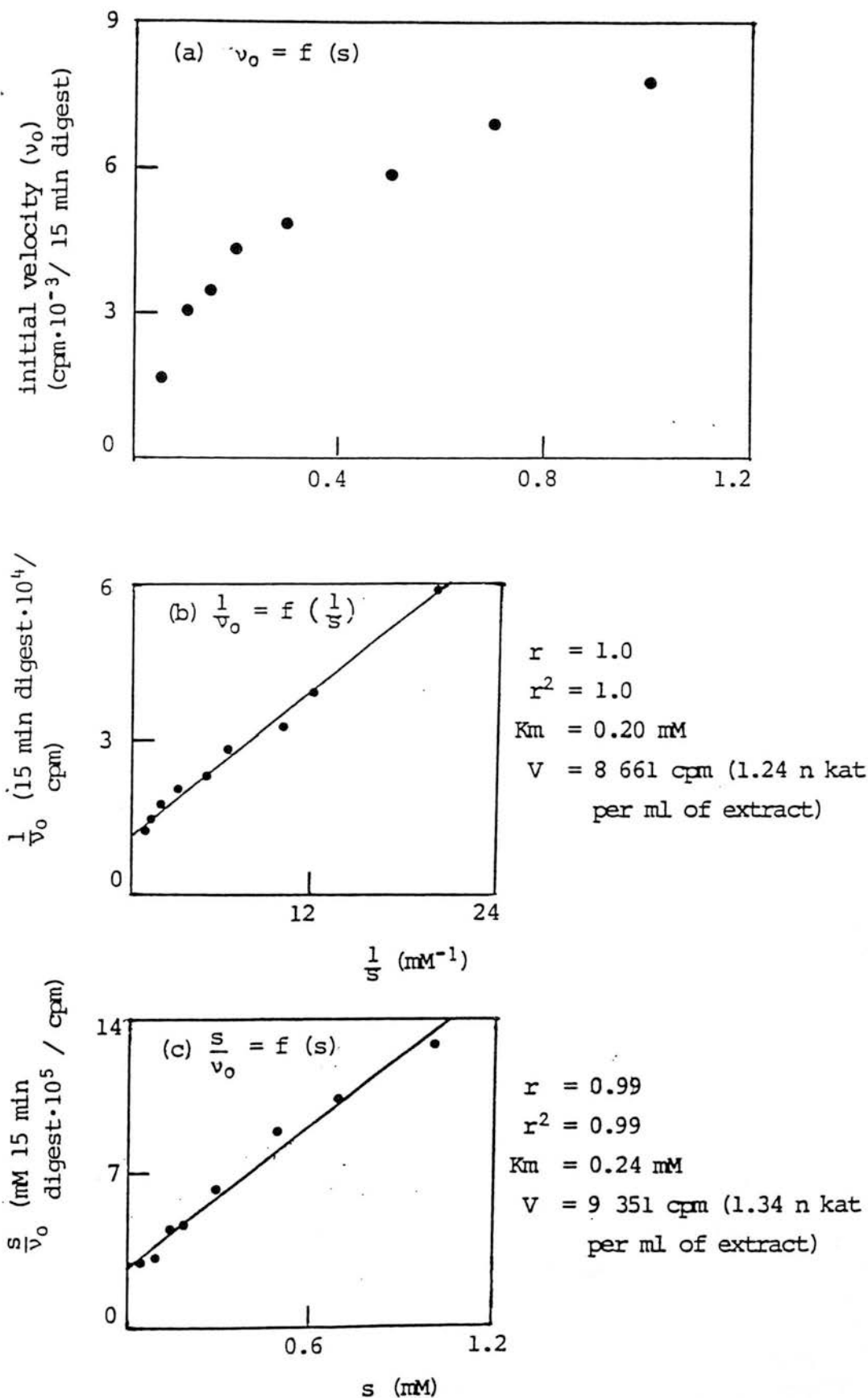


Figure 5.16 3-PGA- dependent leaf ADP- glucose pyrophosphorylase  
initial reaction velocities ([G1P]=0.68mM; [ATP]=1.20mM)

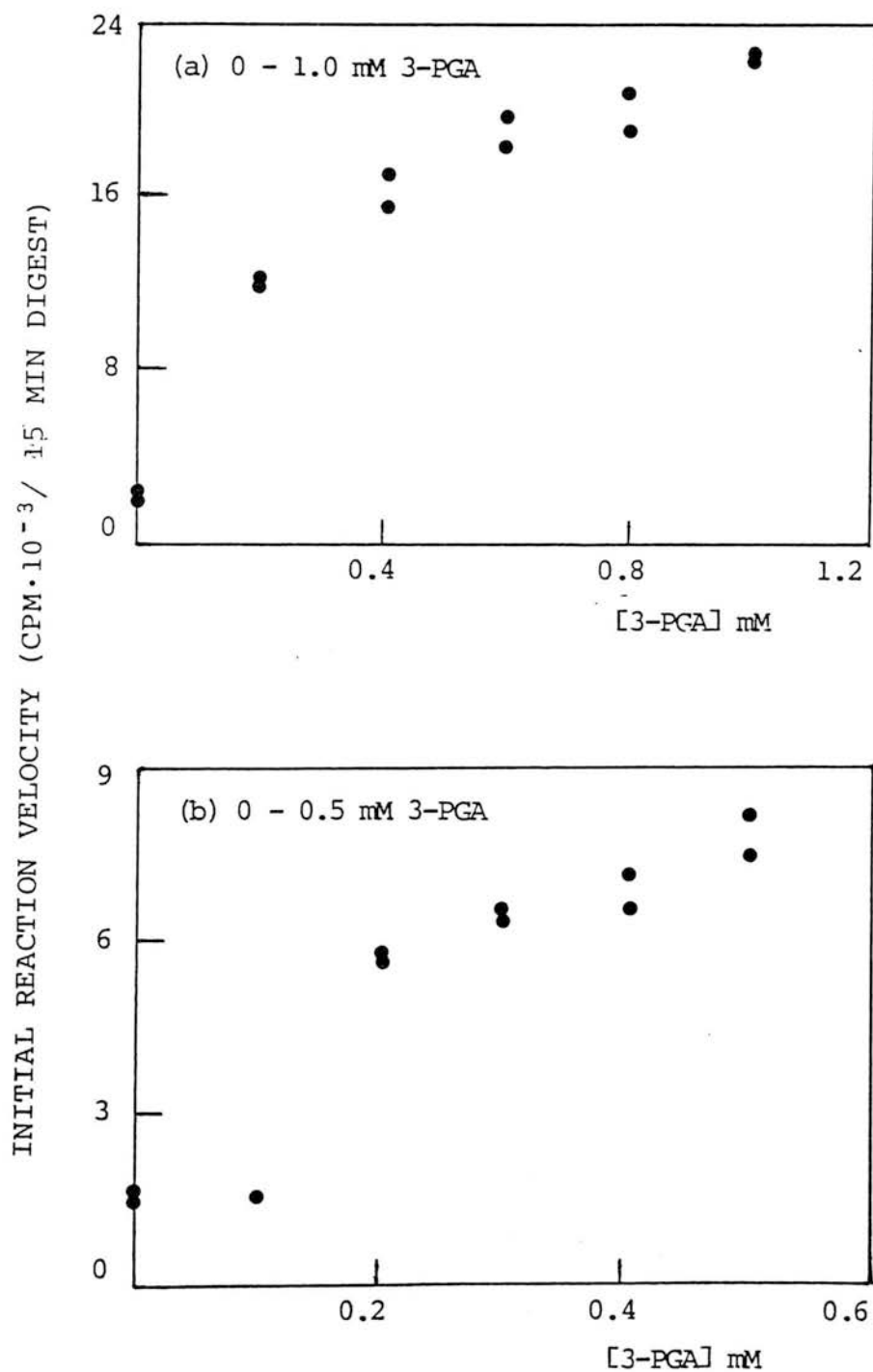


Table 5.7 Initial velocities ( $v_o$ ) leaf ADP - glucose pyrophosphorylase in the absence of 3-PGA as a function of (1) varying G1P concentration (mM) and (2) varying ATP concentration (mM). Digest time 15 min

* G1P (mM)	$v_o$ cpm/15 min	+ ATP (mM)	$v_o$ cpm/15 min
0.02	484	0.05	90
0.04	594	0.10	520
0.08	819	0.15	211
0.12	767	0.20	241
0.16	1078	0.30	577
0.20	1162	0.50	621
0.24	1312	0.70	241
0.28	1521	1.00	508
0.32	1392		
0.42	1721		
0.48	1644		
0.56	1643		

\* ATP constant at 1.20mM

+ G1P constant at 0.68mM

Figs 5.14 and 5.15 show the G1P- and ATP-dependent initial reaction velocities respectively in the presence of 1mM 3-PGA. In both instances the graphs of initial velocities versus substrate concentration (figs 5.14a and 5.15a) show hyperbolic curves which gave linear replots with high positive correlations (figs 5.14b/c and 5.15b/c). The calculated apparent  $K_m$  (G1P) was 0.20mM while  $K_m$  (ATP) was of a similar order (0.20 - 0.24 mM).

The response of the leaf enzyme to activation by 3-PGA, under the conditions of the standard assay (section 5.2.4.1) but with varying concentrations of 3-PGA in the assay digest, is shown in fig 5.16. In fig 5.16a the concentration of 3-PGA was varied between 0 and 1.0m, while in fig 5.16b a narrower range (0 - 0.5mM) was utilised. In both graphs the values of initial velocity in the absence of 3-PGA have been plotted on the respective Y axes. The data show that leaf ADP-glucose pyrophosphorylase was extremely sensitive to activation by 3-PGA and the initial velocities rose rapidly as the concentration of the activator was increased between 0 and 0.5mM.

Estimations of the concentration of 3-PGA required to give 50 per cent activation (data not shown) gave a value of 0.31mM, although it is quite clear from fig 5.16a that the initial velocity was still increasing at 1mM 3-PGA.

### 5.3.11 Contaminating enzymes

Table 5.8 shows the approximate levels of contaminating enzyme activity that were present in both the crude and ammonium sulphate fractionated endosperm and leaf preparations. The results have been expressed in n kat per 5  $\mu$  l of extract and n kat per 10  $\mu$  l of extract for the endosperm and leaf preparations respectively.

Table 5.8 Contaminating enzymes in endosperm and leaf preparations

Enzyme	Activity (n kat)			
	Endosperm (5 $\mu$ l <sup>-1</sup> of extract)		Leaf (10 $\mu$ l <sup>-1</sup> of extract)	
	Crude	40% cut	crude	50% cut
Hexokinase (glucose)	0.70	0.00	0.00	0.00
Hexokinase (fructose)	0.28	0.00	0.00	0.00
G6P dehydrogenase	0.00	0.00	7.04	0.00
Phosphoglucose iso.	13.84	28.14	14.18	45.48
Phosphoglucomutase	0.00	0.00	0.00	0.00
Alkaline phosphatase	3.66	15.04	11.54	30.06
Nucleoside DP kinase	40.27	5.96	113.32	64.46
Phosphorylase	0.28	0.56	0.00	0.00
ADPG pyrophosphatase	0.00	0.00	0.00	0.00
3-PGA kinase	152.31	13.52	NM	213.06
GAP dehydrogenase	112.69	0.00	NM	108.18
3-PGA dehydrogenase	0.00	0.00	NM	0.00
Inorganic PPi'atase	0.36	0.17	NM	0.20

NM = not measured

Phosphoglucose isomerase, alkaline phosphatase, nucleoside diphosphate kinase, 3-PGA kinase and inorganic pyrophosphatase were present in the ammonium sulphate fractions of both the endosperm and the leaf preparations. Of these enzymes, phosphoglucose

Figure 5.17 Effect of varying concentrations of ADP- glucose and PPi on the ATP- dependent activity of endosperm ADP- glucose pyrophosphorylase

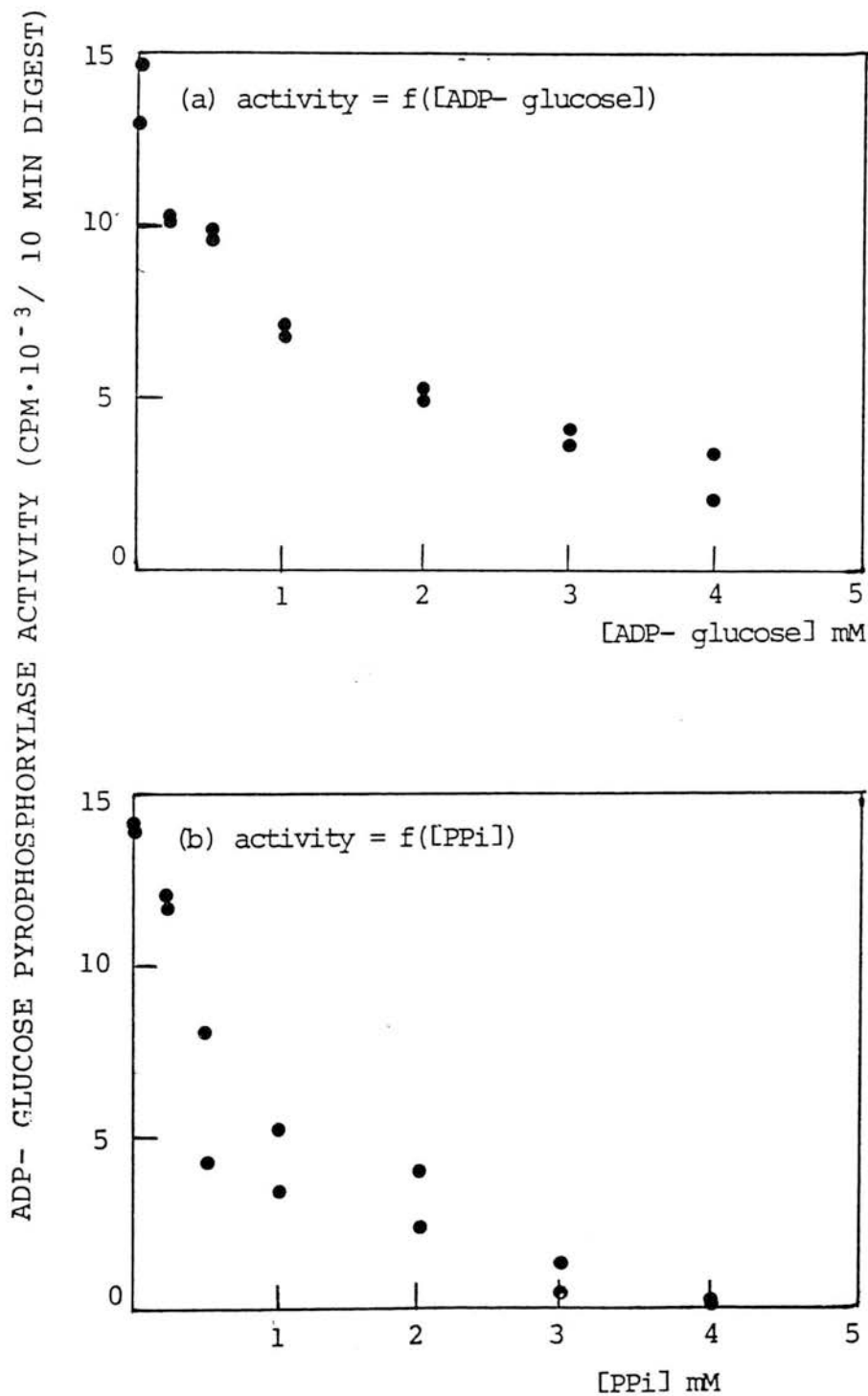


Figure 5.18 Effect of varying concentrations of Pi on the ATP-dependent activity of endosperm ADP- glucose pyrophosphorylase

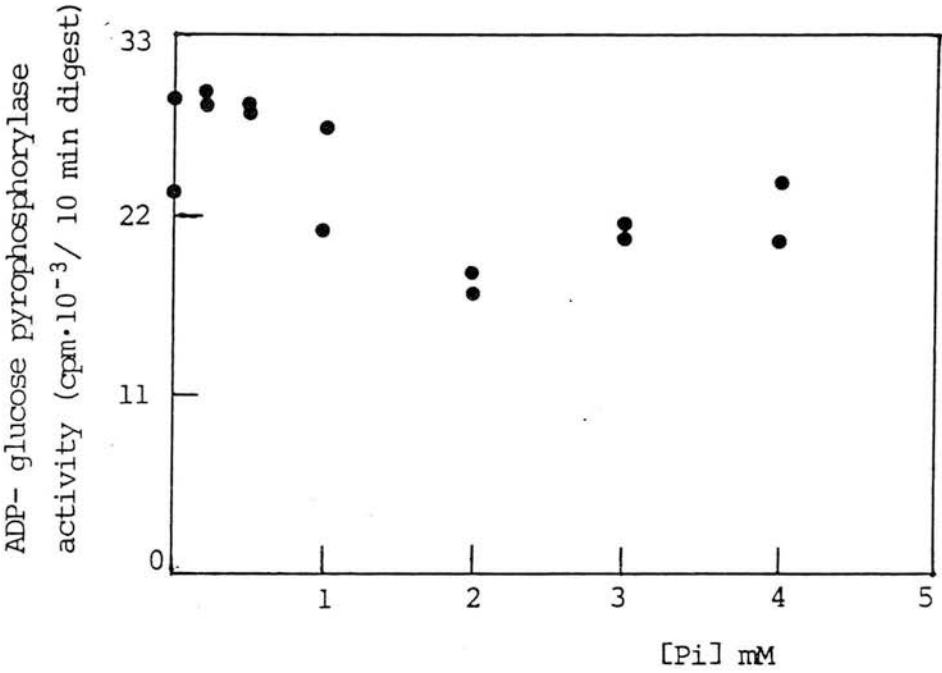
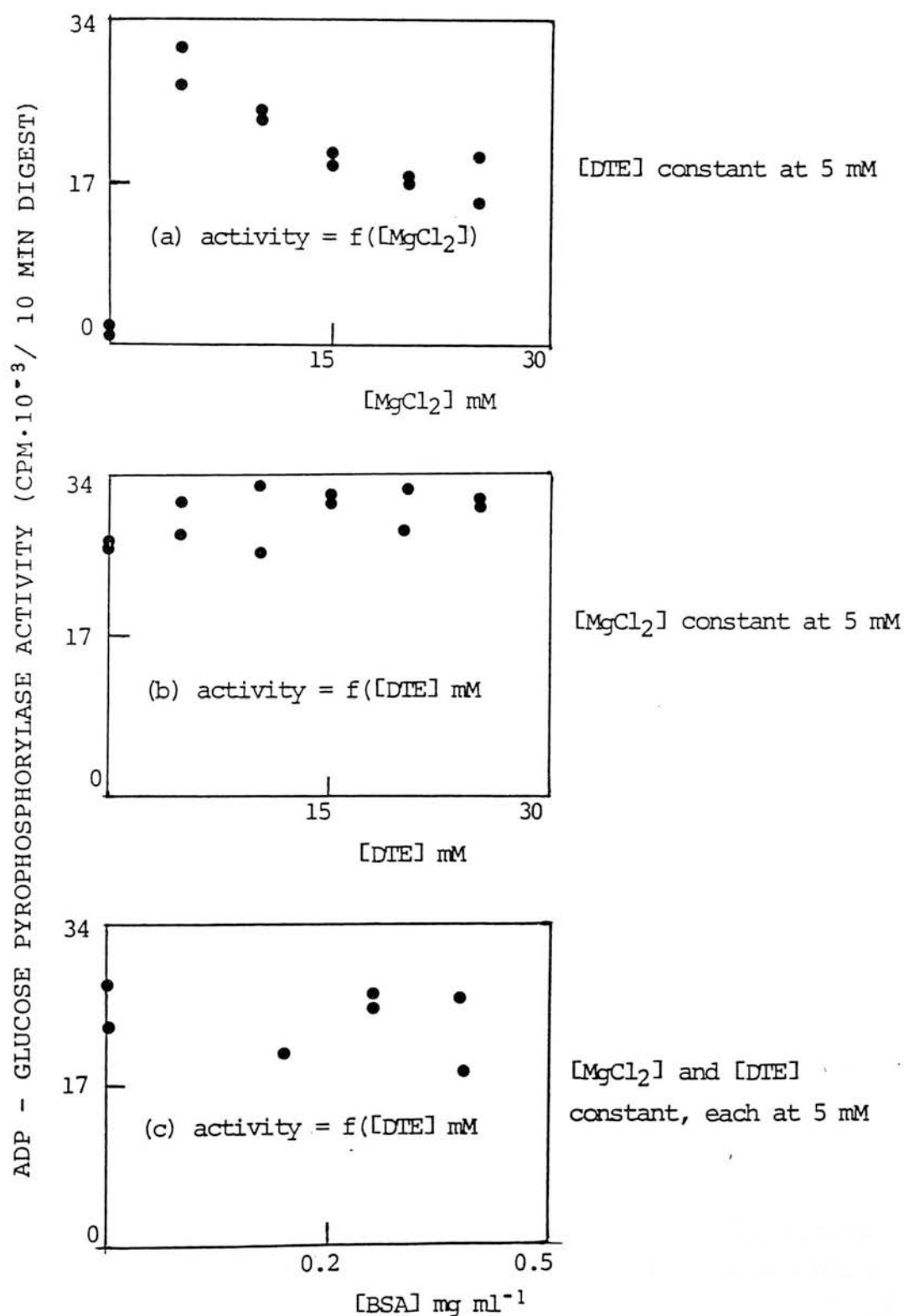




Figure 5.19 Effect of varying concentrations of  $\text{MgCl}_2$ , DTE <sup>151</sup>. and BSA on the ATP- dependent activity of endosperm ADP- glucose pyrophosphorylase



isomerase and alkaline phosphatase were increased in activity by ammonium sulphate fractionation i.e. relative to the fractions used for the ADP-glucose pyrophosphorylase enzyme studies. 3-PGA kinase and inorganic pyrophosphatase were not assayed in the crude leaf preparation. Phosphorylase activity was only detected in the endosperm preparations and was increased 2 times by ammonium sulphate fractionation. Glyceraldehyde phosphate dehydrogenase had significant levels of activity in the leaf ammonium sulphate fraction, as did 3-PGA kinase.

#### 5.3.12 The effect of ADP-glucose, P<sub>PP</sub>i and P<sub>i</sub> on the activity of endosperm ADP-glucose pyrophosphorylase

Fig 5.17 shows the effect of increasing concentrations of ADP-glucose (fig 5.17a) and P<sub>PP</sub>i (fig 5.17b) on the ATP-dependent activity of endosperm ADP-glucose pyrophosphorylase. Both the metabolites had similar effects upon the enzyme activity, reducing formation of ADP- [U-<sup>14</sup>C] glucose. Within the concentration range that was studied, P<sub>PP</sub>i was the more effective inhibitor, completely inhibiting product formation at 4 mM.

The effect of P<sub>i</sub> on the activity of endosperm ADP-glucose pyrophosphorylase was quite different (fig 5.18) and there was little inhibition within the variation of the duplicate analyses.

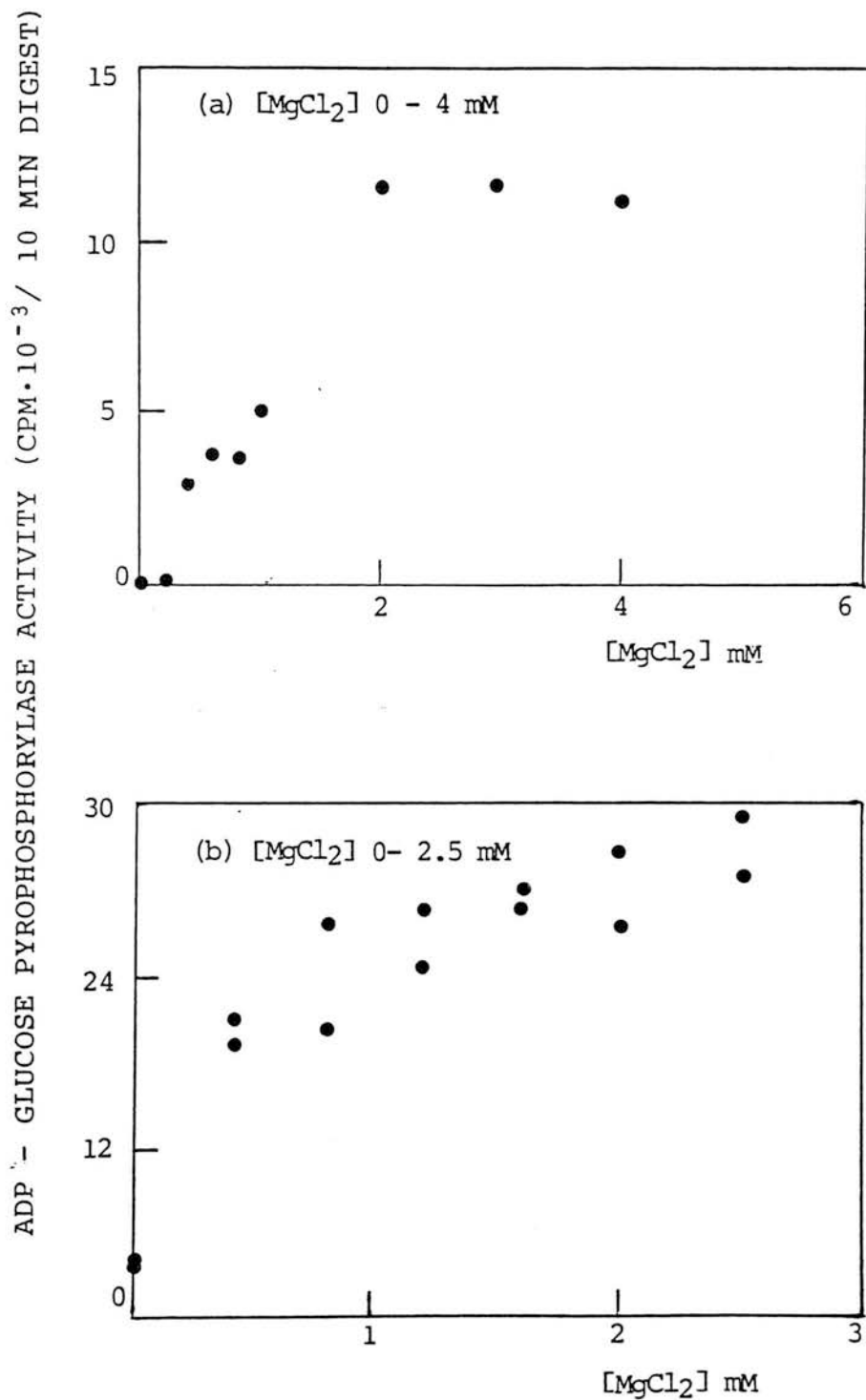
#### 5.3.13 The effect of MgCl<sub>2</sub>, DTE and BSA on endosperm ADP-glucose pyrophosphorylase activity

Figure 5.19 shows the activity of endosperm ADP-glucose pyrophosphorylase in response to increasing concentrations of MgCl<sub>2</sub>, DTE and BSA in the assay digest.

MgCl<sub>2</sub> was essential for ATP-dependent activity although there was a fall off as the concentration in the digest increased beyond 5 mM.

Increasing concentrations of DTE in the presence of MgCl<sub>2</sub> (5 mM) had little effect on enzyme activity although there may have been

Figure 5.20 Effect of varying concentrations of  $\text{MgCl}_2$  on the ATP-dependent activity of endosperm ADP- glucose pyrophosphorylase



a slight stabilising effect on the time - dependent decrease in ADP-glucose pyrophosphorylase activity (section 5.3.8).

BSA had no effect on the activity of the enzyme and was difficult to evaluate since the boiling (45 s) step in the assay procedure caused protein precipitation, resulting in difficulties in transferring aliquots of the digest to the paper strips.

The dependence on  $\text{MgCl}_2$  for efficient rates of endosperm ADP-glucose pyrophosphorylase activity was further investigated by varying  $\text{MgCl}_2$  concentrations in the enzyme digest between 0 and 4mM (fig 5.20a) and from 0 to 2.5mM (fig 5.20b). Maximum activity was attained in the presence of 2-3mM  $\text{MgCl}_2$  (fig 5.20a) while the most rapid rise in activity occurred between 0 and 0.5mM  $\text{MgCl}_2$  (fig 5.20b).

#### 5.3.14 Activity of endosperm ADP-glucose pyrophosphorylase in the presence of various salts

Aliquots of dialysed endosperm ADP-glucose pyrophosphorylase were incubated with Pi, Pi/ $\text{MgCl}_2$ ,  $\text{MgCl}_2$  and NaCl as described in section 5.2.11.9. The total added salt concentration of each system was 4mM. A control aliquot with water substituting for the salts was also set up. The control aliquot was assayed immediately and in triplicate for enzyme activity and the average value computed as time zero ( $T_0$ ) in table 5.9. The control aliquot, together with the 4 salt-treated aliquots, was re-assayed in duplicate 16 h later ( $T_{16}$ ). The average values were expressed as a percentage of the control  $T_0$  value. In both salt systems where Pi was present (table 5.9) the activity at  $T_{16}$  was highest. In particular, the aliquot which contained 4mM  $\text{Na}_2\text{HPO}_4$  retained nearly 60 per cent of the control  $T_0$  value. In contrast the water control at  $T_{16}$  was found to be 42 per cent of its respective  $T_0$  value.

A second experiment was designed where aliquots of the ammonium sulphate preparation were dialysed against homogenisation buffer

Figure 5.21 Stability of endosperm ADP- glucose pyrophosphorylase<sup>155</sup> following dialysis in the presence of various salts

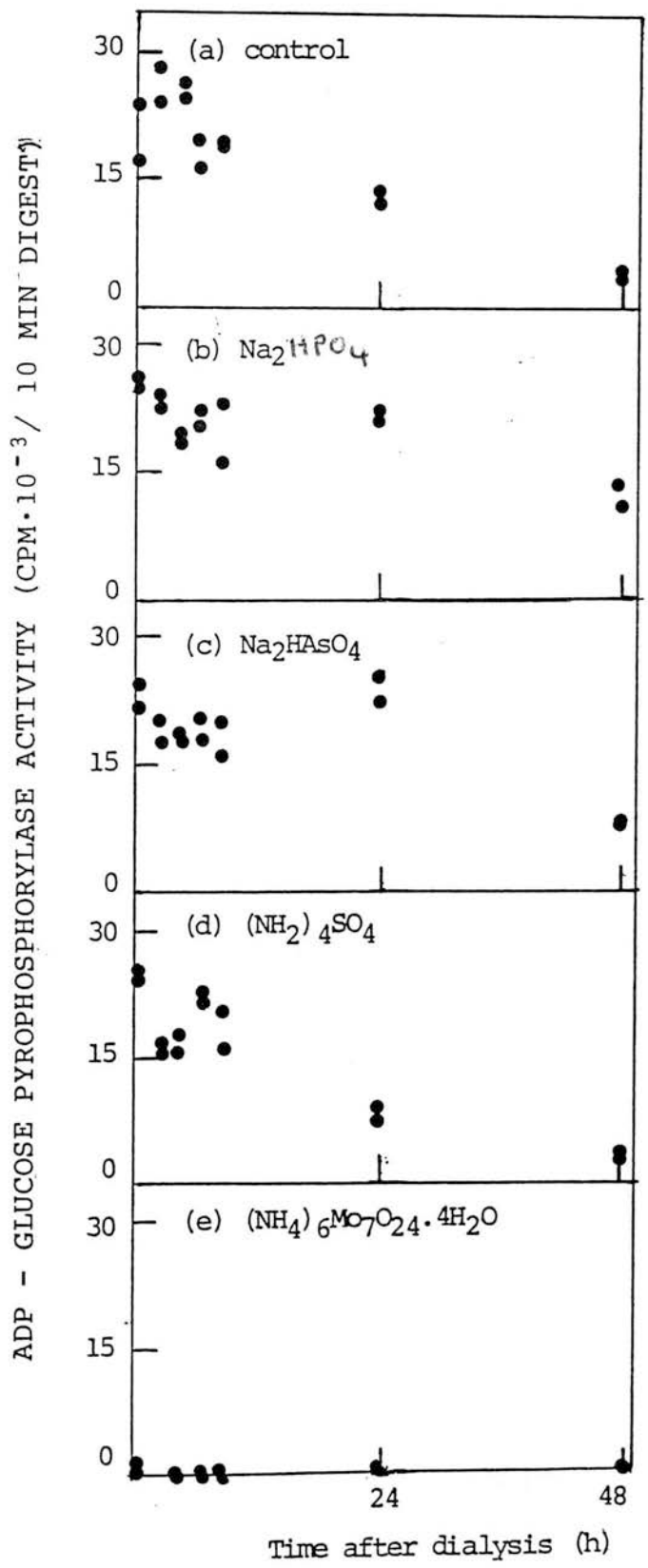


Table 5.9 Time-dependent activity of dialysed endosperm ADP-glucose pyrophosphorylase in the presence of various salts

Sample	Activity (cpm/ 10 min digest)	$\bar{x}$ activity (cpm/10 min digest)	% of control $T_0$ activity
Control $T_0$	12 918, 12 349, 12 913	12 827	100
Control $T_{16}$	5 474, 5 191	5 333	42
(at $T_{16}$ ) $\text{Na}_2\text{HPO}_4$	7 706, 7 320	7 513	59
$\text{Na}_2\text{HPO}_4/\text{MgCl}_2$	6 446, 7 140	6 793	53
$\text{MgCl}_2$	5 545, 4 711	5 128	40
$\text{NaCl}$	6 222, 5 703	5 963	46

Key:  $T_0$  = time zero for water treated control

$T_{16}$  = time 16 h for all the samples.

containing phosphate, arsenate, sulphate and molybdate ions respectively. Following dialysis, aliquots (5  $\mu$ l) of each dialysate, including a control containing homogenisation buffer only, were assayed in duplicate over a 48 h period. The results are given in fig 5.21.

The analyses of dialysate aliquots up to 8 h showed similar activities except for the dialysate containing molybdate anions (fig 5.21e) which had no detectable activity throughout this period and indeed for the remainder of the time course. At the 24 h mark, the highest activities were recorded in the samples containing phosphate (fig 5.21b) and arsenate (fig 5.21c) anions respectively

and this pattern was repeated at 48 h. At this time the sample containing phosphate (fig 5.21b) retained approximately 47 per cent of its original measured activity, while arsenate, sulphate and the buffer control (figs 5.21c, 5.21d and 5.21a) had determined activities approximating to 33, 10 and 28 per cent of their respective original levels.

#### 5.4 Discussion

##### 5.4.1 The radiochemical assay for the determination of ADP- and UDP-glucose pyrophosphorylase activities

The radiochemical assay was used routinely throughout the course of this work for the determination of ADP- and UDP- glucose pyrophosphorylase activities. It was shown to be extremely reliable, however there were a number of potential problems which required careful consideration and these merit some discussion.

The method of strip counting on DEAE-cellulose paper strips was dependent upon the binding capacity of the paper strips. Salt concentrations in excess of 10mM progressively reduced the retention characteristics of the strip. For this reason it was important that enzyme preparations containing high concentrations of salts, e.g. ammonium sulphate, were dialysed essentially free of these salts prior to incorporation in the assay digest. Similar phenomena were reported for DEAE-cellulose paper disc techniques (Roberts and Tovey, 1970). The original strip counting method (Sherman, 1963) used 8 x 1.5 cm DEAE-cellulose strips and these dimensions were used in this work. However Shen and Preiss (1964), and subsequent workers referring to their method, used smaller strips (8 x 1cm). Preliminary experiments in this work indicated that the larger strip area was required.

A large number of alkaline phosphatase preparations are commercially available but many of these have contaminating enzyme activities. In particular the presence of hydrolytic activities

with respect to nucleoside diphosphate sugars have been reported (P. Keeling, personal communication). The preparation used in this work (E.Coli alkaline phosphatase) had no such detectable activities.

The retention and elution efficiencies were in agreement with similar parameters published by the original authors of this method (Sherman, 1963; Shen and Preiss, 1964) and also with more recent studies (Fuchs and Smith, 1979). The elution efficiencies of the unreacted  $[U-^{14}C]$  G1P reported in table 5.2 were the minimum levels recorded for the control procedures. In practice, approximately 99.8 per cent of unreacted  $[U-^{14}C]$  G1P was regularly eluted by the alkaline phosphatase digest and the consecutive strip swirling technique.

The use of PBD as the primary fluor (Peng, 1977) gave improved counting efficiencies with respect to the more commonly used 2,5-diphenyloxazole (PPO).

In general, the vast majority of the data was expressed in cpm per time (min) of assay digest. The equations for conversion to units of enzyme activity may introduce degrees of error and were only used for the developmental studies and in the kinetics section for the determination of maximum initial reaction velocities.

Identification of radiolabelled reaction products confirmed the specificity of the radiochemical assay and showed that there was no detectable subsequent transformation of nucleoside diphosphate glucose by, for example, epimerases or dehydrogenases (Feingold, 1982). Additionally there was no evidence of any interconversion between ADP-  $[U-^{14}C]$  glucose and UDP -  $[U-^{14}C]$  glucose in the presence of the respective pyrophosphorylases.

#### 5.4.2 Developmental studies

The activities of ADP- and UDP- glucose pyrophosphorylases in



developing endosperms varied both in developmental profiles and in relative catalytic rates. The ATP-dependent activity rose very rapidly from a relatively low base level, while the rate of increase of the UTP-dependent activity was much slower but commenced from a higher initial catalytic rate. The respective rates of decline were similar, but substantial levels of UDP-glucose pyrophosphorylase activity were still present at 50 days after anthesis. The rapid rise in ATP-dependent activity with respect to the more gradual rise in UTP-dependent activity was reflected by the steep decline in relative activities that was observed during the early stages of endosperm development.

The data in this work have been derived from extracts of endosperm tissue only, except for the determination of ATP- and UDP-dependent activities at 50 days after anthesis where caryopses, minus their embryos, were used. At these later stages of development it was not possible to separate efficiently the pericarp/testa from the endosperm tissue, hence the use of the de-embryonated caryopses. Turner (1969) and more recently Kumar and Singh (1980) have published results on pyrophosphorylase activities in developing wheat. However in both studies whole wheat grains, i.e. caryopses, were used and the methods of assay to determine pyrophosphorylase activities did so in the direction of GlP synthesis. These are scarcely valid procedures since in the first instance there is no recognition of the tissue morphology of the wheat caryopsis and in the second instance, at least for ADP-glucose pyrophosphorylase, the proposed catalytic role of the enzyme, in sucrose-starch conversion, is in the direction of ADP-glucose synthesis (Preiss et al., 1985).

In this work, ADP-glucose pyrophosphorylase followed the same activity profile as was observed for endosperm sucrose synthase (section 4.3). Both these enzymes rose steadily from low levels at the beginning of endosperm development, reaching apparent maximum catalytic rates during the period of rapid dry weight accumulation (section 2.3). The activities of the two enzymes then declined,

almost in parallel, although the drop in ADP-glucose pyrophosphorylase activity was less rapid. The peak of ADP-glucose pyrophosphorylase activity occurred slightly later than for sucrose synthase activity, however it is probable that these differences merely reflect errors in morphological ageing (section 2.4). Similar patterns of coincident sucrose synthase and ADP-glucose pyrophosphorylase maximum activities have been observed in maize endosperms (Tsai et al., 1970), rice grains (Perez et al., 1975) and in mung beans (Tsai et al., 1983).

The developmental patterns of endosperm sucrose synthase and ADP-glucose pyrophosphorylase suggest that their activities may be significant in regulating the rates of starch biosynthesis in wheat. Reeves et al. (1986) have measured mRNA for ADP-glucose pyrophosphorylase during wheat endosperm development and the elaboration of ADP-glucose pyrophosphorylase polypeptides over the same period. The data show that transcription of the ADP-glucose pyrophosphorylase gene(s) occurs just prior to the onset of rapid starch accumulation and that translation of the messenger, resulting in production of ADP-glucose pyrophosphorylase, occurred consecutively. Control of ADP-glucose pyrophosphorylase biosynthesis would therefore seem to be at the level of gene transcription since there was no evidence of translational control. The levels of ADP-glucose pyrophosphorylase polypeptide declined in the later phases of development and this points to a specific protein/enzyme degradation rather than an inactivation. These data are complementary to the general findings presented in this work, that ADP-glucose is a developmental enzyme, the synthesis of which is apparently linked to the onset and subsequent rapid accumulation of endosperm dry weight. Earlier mutant studies in maize (Tsai and Nelson, 1966; Dickinson and Preiss, 1969b) implicated relative lowered levels of ADP-glucose pyrophosphorylase activity as being responsible for poorly developed endosperms in the shrunken-2 maize mutant. These studies together with current developments suggest an important role for ADP-glucose pyrophosphorylase in the regulation of starch biosynthesis. However the mode of regulation in wheat

endosperms may not be that of a fine control by metabolic intermediates, as has been claimed for 'all' ADP-glucose pyrophosphorylases in plant cells (Preiss, et al., 1985) but that of a coarse control mechanism at the level of protein biosynthesis.

In addition to the similar developmental patterns observed during the course of the present work for wheat endosperm sucrose synthase and ADP-glucose pyrophosphorylase activities, their respective determined catalytic rates were essentially the same. In consideration of endosperm dry weight accumulation, these rates were apparently more than adequate (section 4.4.1), although in the case of sucrose synthase the metabolite data (section 3.3.1), together with the measurements of UDP- and sucrose-dependent initial reaction velocities (section 4.4.2), implied a much lower in vivo activity. With respect to ADP-glucose pyrophosphorylase, the studies on initial reaction velocities (G1P- and ATP-dependent) in relation at least to estimated levels of G1P (section 3.4.2) did not suggest that this substrate was rate limiting (see section 5.4.3).

The observed levels of UDP-glucose pyrophosphorylase activity during the period of rapid dry weight accumulation were approximately 5-7 times higher than either ADP-glucose pyrophosphorylase or sucrose synthase. Turner and Turner (1975) have suggested that increased levels of UDP-glucose pyrophosphorylase in wheat may be responsible for the catabolism of UDP-glucose rather than its biosynthesis (see section 3.4.2). The data in section 3.3.1 show levels of UDP-glucose that were twice that found for ADP-glucose. These levels of UDP-glucose may have been a consequence of increasing UDP-dependent sucrose synthase activity (section 4.3.1). UTP-dependent UDP-glucose pyrophosphorylase activity has been shown to be subject to strong product inhibition by UDP-glucose (Turnquist and Hansen, 1973; Turner and Turner, 1975; Feingold, 1982), although most of the evidence derives from work done on animal tissues. Plant tissue UDP-glucose pyrophosphorylases have reported UDP-glucose inhibitor

constants ( $I_{0.5}$ , where  $I_{0.5}$  is the concentration of inhibitor observed to reduce maximum initial velocity by 50 per cent) of between 0.05 and 0.16 mM (Hopper and Dickinson, 1972; Turnquist and Hansen, 1973). In the work reported in this thesis, the levels of UDP-glucose found in wheat endosperms during the period of rapid dry weight accumulation were between 4 and 6 n moles per endosperm. This may represent a concentration of between 0.3 and 0.5mM based on endosperm water content (results not shown) which might be sufficient to inhibit the UTP-dependent UDP-glucose pyrophosphorylase reaction. Such a phenomenon could be compatible with starch biosynthesis, since the reversal of the UDP-glucose pyrophosphorylase reaction (cleavage of UDP-glucose) would bring about the generation of GlP which might then be utilised by ADP-glucose pyrophosphorylase for the synthesis of ADP-glucose. Clearly this sequence of events would depend upon the activity of sucrose synthase (UDP-dependent activity) and the availability of PPi.

#### 5.4.3 Studies on endosperm and leaf pyrophosphorylases

Ammonium sulphate precipitation was shown to be a satisfactory initial purification procedure for both endosperm (ADP- and UDP-glucose) and leaf (ADP-glucose) pyrophosphorylases from wheat. This procedure had been previously used by Tovey and Roberts (1970) for the preparation of ADP- and UDP- glucose pyrophosphorylases from whole wheat flour. These workers used broad 0-60 per cent saturation ammonium sulphate cuts, while the results reported in this thesis were, in the main, obtained by narrow incremental ammonium sulphate fractionation (Scopes, 1984a), increasing the per cent saturation ammonium sulphate by 10 per cent at each consecutive stage. This procedure gave a reasonable crude separation of the major ATP- and UTP-dependent activities in the endosperm preparations, although there was significant UTP-dependent activity in the 30-40 and 40-50 per cent saturation ammonium sulphate cuts. This activity was lowest in the 30-40 per cent cut. This fraction was used throughout the course of this work

for analyses of wheat endosperm ADP-glucose pyrophosphorylase activity. Various workers investigating other plant tissue ADP-glucose pyrophosphorylases have used similar preparations notably for sweet corn (Amir and Cherry, 1972) and potato (Sowokinos and Preiss, 1982), although in both these instances further chromatographic procedures were used.

Tovey and Roberts (1970) have stated that 90 per cent of both ADP- and UDP-glucose pyrophosphorylase activities from wheat flour was precipitated by 60 per cent saturated ammonium sulphate. With the endosperm preparations that were fractionated in this work, approximately 55 per cent of the total pyrophosphorylase activity (ATP- and UTP- dependent) was obtained above 60 per cent saturated ammonium sulphate and this portion of the activity was exclusively UTP-dependent.

Leaf ADP-glucose pyrophosphorylase was prepared by essentially the same procedures and ATP-dependent activity was found mainly in the 40-50 per cent saturated ammonium sulphate fraction. This was in agreement with the results published by McDonald and Strobel (1970) for wheat leaf ADP-glucose pyrophosphorylase and also with reported data for the same enzyme in sorghum and barley leaf which was precipitated by 0-59 and 0-40 per cent saturated ammonium sulphate respectively (Sanwal *et al.*, 1968). UTP-dependent activity was also present in the wheat leaf preparation but this was not quantified.

The radiochemical assay digest contained 0.05  $\mu$  Ci of radioactivity prior to initiation of either the ADP- or UDP- glucose pyrophosphorylase reaction. This gave a quenched count of approximately  $77 \times 10^3$  cpm. It was therefore important to ensure that the G1P-dependent pyrophosphorylase reactions utilised [ $U-^{14}C$ ] G1P in such a manner as to maintain a linear rate of radiolabel incorporation over the digest time without having to increase the specific activity of the digest. Both UTP- and ATP- dependent activities from endosperm and leaf preparations respectively were essentially linear for up to 20 min. This was also true for the

latter activity in the presence of 3-PGA. However linearity was much harder to maintain with endosperm ADP-glucose pyrophosphorylase. These findings apparently disagreed with those of Tovey and Roberts (1970) who reported that the assay of UDP-glucose pyrophosphorylase was more susceptible to deviations from linearity. It is difficult to make a proper comparison between these sets of data for two reasons. The first is that Tovey and Roberts (1970) used whole wheat flour preparations containing both embryo and pericarp/testa tissues as well as endosperm material. The second is a consequence of the methods used in this work which measured UTP-dependent activity as a contaminating activity in the 30-40 per cent ammonium sulphate cut. Clearly there is a possibility that the UTP-dependent activity in the 60-70 per cent saturation ammonium sulphate fraction may have caused similar problems to those observed by Tovey and Roberts (1970).

The pH optimum for endosperm ADP-glucose pyrophosphorylase was in general agreement with pH optima previously determined for this enzyme from wheat flour (Espada, 1962; Tovey and Roberts, 1970) and from reserve tissues of other plants including maize endosperm (Dickinson and Preiss, 1969a), sweet corn (Amir and Cherry, 1972) and potato tubers (Sowokinos, 1981).

HEPES buffer produces the highest apparent relative activity for this enzyme and this result was consistent with the data reported for sweet corn ADP-glucose pyrophosphorylase (Amir and Cherry, 1972). Similar pH optima were observed for the UTP-dependent activity in the endosperm preparation and for leaf ADP-glucose pyrophosphorylase, although this latter activity was only determined in the absence of 3-PGA.

The thermal characteristics of the endosperm ATP- and UTP-dependent activities in the 30-40 per cent cut were interesting in that while the ATP- dependent activity dropped with respect to temperature and incubation time it appeared slightly more heat stable than the UTP-dependent activity. This was surprising in view of the apparent



instability of the dialysed preparation. Preiss et al. (1971) have published results which compared the heat stabilities of maize embryo and endosperm enzymes, the former enzyme retaining a high degree of stability at 65°C. While these data are not directly comparable with the findings discussed in this work, they demonstrate the importance of accurate description of the tissues used for analysis. Much of the published material on the regulation of starch biosynthesis in cereal 'endosperms' relates not to cereal endosperms but to whole grains or caryopses. It is also relevant to point out that many of these enzymes have been assayed at temperatures optimal for animals rather than for plants i.e. at 37°C.

Attempts to purify further the ammonium sulphate preparations by gel permeation and ion exchange chromatography were unsuccessful, although the UTP-dependent activity chromatographed particularly well on the ion exchange column. These difficulties with the purification of wheat ADP- glucose pyrophosphorylases have been previously reported (Espada, 1962; Tovey and Roberts, 1970) and were believed to be related to a loss of activity as a consequence of desalting occurring during the chromatographic procedures. In this work it was found that the enzyme activity in the crude preparation, i.e. as extracted by the homogenisation buffer, was stable and the components of this buffer were incorporated in both the gel permeation and ion-exchange procedures. Even with these modifications it was not possible to elute active fractions. Clearly the approximate 4 h half-life of the dialysed extract was too short to allow successful chromatography, particularly for the ion-exchange procedures which were carried out over 35 h. The limited success in separating active ADP-glucose and UDP-glucose pyrophosphorylases from wheat flour reported by Espada (1962) may have been due to a contribution from the embryo rather than the endosperm. Alternatively ADP-glucose pyrophosphorylase activity present in wheat flour may be more stable than the activity present in the developing endosperms.

The regulation of starch biosynthesis in leaves has been assigned, in the main, to the regulatory properties of leaf ADP-glucose pyrophosphorylases and these properties have been extensively reviewed over the past few years (Preiss and Levi, 1979; Preiss, 1982a and 1982b; Preiss, 1984; James *et al.*, 1985). In the work reported here, a large number of known plant metabolites and other chemicals were tested with both the endosperm and leaf preparations for possible effects on the activity of the respective ADP-glucose pyrophosphorylases. The leaf enzyme responded to activation by 3-PGA, 2-PGA, G6P, F6P and PEP in the manner typical of leaf ADP-glucose pyrophosphorylases as originally described for spinach leaf (Ghosh and Preiss, 1966) and as demonstrated in a variety of leaf tissues (Sanwal *et al.*, 1968). This data was also in general agreement with that previously published for wheat leaves (McDonald and Stobel, 1970), although in that instance the enzyme was assayed in the direction of ATP synthesis. The leaf enzyme was strongly inhibited by PPi and Pi and also to a lesser extent by AMP, ADP and ADP-glucose. These data are also in agreement with previous work as described above.

In contrast, the enzyme from endosperm tissue did not exhibit any of the activation characteristics that were observed for the leaf enzyme. Dickinson and Preiss (1969a) have reported that 3-PGA stimulated the activity of maize endosperm ADP-glucose pyrophosphorylase by approximately 4 times the control activity. However a relatively high concentration (10mM) of 3-PGA was required to produce this activation effect. This high concentration of 3-PGA might have affected the efficiency of alkaline phosphatase in the assay digest by competing with unreacted  $[U-^{14}C]$  G1P and may have given increased counts on the DEAE-cellulose strips. It is not clear from the methodology whether this phenomenon was examined. During the course of the work reported here it was found that for efficient hydrolysis of unreacted  $[U-^{14}C]$  G1P, an alkaline phosphatase digest time of 1.5h was required and this was in the absence of any added 3-PGA. Sowokinos and Preiss (1982) found that potato tuber ADP-glucose pyrophosphorylase activity was



stimulated approximately 33 times at 37°C in the presence of 3mM 3-PGA. However while potato tubers are clearly reserve tissues, they are not truly non-chlorophyllous reserve tissues since they have the capacity, under certain circumstances, for photosynthesis.

The inhibition of the endosperm enzyme by EDTA may have been due to a sequestration of ions essential for the activity of this enzyme. The data determined in this work show that  $MgCl_2$  was essential for the activity of endosperm ADP-glucose pyrophosphorylase and the effect of EDTA may have been to reduce the levels of participating  $Mg^{++}$  ions. Since these ions were present at a concentration of 5mM in the assay digest (section 5.2.4.1) and the concentration of EDTA was 1mM (section 5.2.11.6), higher levels of EDTA would probably be necessary for complete inhibition. Interestingly EDTA did not have a significant inhibitory effect on the leaf enzyme and while this aspect of the data was not investigated further, it is perhaps important to note that Sanwal *et al.* (1968) included EDTA, at 1-2mM, in all the homogenisation and dialysis media used in the partial purification (by ammonium sulphate fractionation) of various leaf ADP-glucose pyrophosphorylases. Although subsequent assay procedures were carried out in the absence of EDTA, the inclusion of the chelating agent up to this point was apparently not detrimental to respective enzyme activities.

ADP inhibited both the endosperm and leaf enzyme, as did ADP-glucose. This latter inhibition was not unexpected, since ADP-glucose should act to prevent the formation of ADP [U- $^{14}C$ ] glucose by product inhibition of the ATP-dependent pyrophosphorylase reaction. The effect of ADP might be a function of common molecular groups, in particular the pyrophosphate group which has been implicated in the binding sites (Turnquist and Hansen, 1973; Feingold, 1982) for the nucleoside triphosphate. However the evidence for this is extremely limited and derives mainly from studies on UDP-glucose pyrophosphorylase. Measurement of ADP levels in developing endosperms (section 3.3.1) showed that the highest amounts were determined during the period of rapid dry

weight accumulation. This might be significant with respect to ADP-glucose pyrophosphorylase activity if all of the amounts were able, directly, to influence the enzyme. Clearly, in the absence of information on compartmentalisation, it is difficult to assign the observed inhibitory effect of ADP to a definite role in the regulation of endosperm ADP-glucose pyrophosphorylase. However, since ADP is a product of the ADP-glucose-dependent starch synthase reaction, then elevated levels, perhaps due to a reduction of its rate of removal, e.g. for the synthesis of ATP, may cause an inhibition of starch synthesis. This inhibition might be due to a reduction in the synthesis of ADP-glucose by a substrate (ATP) limiting situation rather than a specific inhibition.

The effects of the presence of ADP-glucose and PPI on the activity of ADP-glucose pyrophosphorylase can be assigned to product inhibition and the data show evidence of this phenomenon. These data were confirmed by later results where the digest concentration of these metabolites was increased to 4mM respectively.

G1P- and ATP- dependent initial reaction velocities were estimated by the methods previously discussed in section 4.4.2.1. The general range of the apparent  $K_m$  (G1P) values for endosperm ADP-glucose pyrophosphorylase was found to be higher than that previously reported by Tovey and Roberts (1970), who quoted a value of 0.04mM.

However since these workers did not show the data from which that value was determined it is impossible to make a proper comparison. In the present work the initial data gave a calculated  $K_m$  (G1P) of approximately 0.08mM. When this parameter was re-determined over a narrow concentration range of G1P (0-0.28mM), the value was found to be 0.30mM. This figure may have been overestimated due to 2 relatively high initial reaction velocities obtained at 0.28mM G1P. When these values were excluded from the Hanes plot, the calculated  $K_m$ (G1P) fell to 0.25mM. The endosperm concentration of G1P was estimated to be at least 0.65mM and may have been as high as 1.30mM (section 3.4.2). This estimation does not take into account the

apparent low recovery rate of G1P (66 per cent). Clearly these values suggest that G1P was not rate-limiting with respect to endosperm ADP-glucose pyrophosphorylase activity, although, as has been mentioned previously, consideration of compartmentalisation may affect these findings. There was no evidence of sigmoidal kinetics and the curve of initial reaction velocity versus substrate (G1P) concentration followed a hyperbolic function. The values for initial reaction velocities, obtained between 0 and 0.28mM G1P, may have been affected by levels of alkaline phosphatase activity which was found to be contaminant of the 40 per cent ammonium sulphate endosperm fraction. However these levels were estimated under optimum assay conditions for alkaline phosphatase (section 5.2.4.4) and would have been considerably less during the assay digest for ADP-glucose pyrophosphorylase (section 5.2.4.1).

The calculated  $K_m$  (G1P) for leaf ADP-glucose pyrophosphorylase was of a similar order to that obtained for the endosperm enzyme, however the value of 0.20mM was dependent upon the presence of 3-PGA (1mM). The graph of initial velocity versus G1P concentration was hyperbolic and was apparently not affected by contaminating levels of alkaline phosphatase.

Clearly a major difference between wheat endosperm and wheat leaf wheat ADP-glucose pyrophosphorylases is the effect of 3-PGA. The leaf enzyme activity was essentially dependent upon the presence of this activator for comparable rates of ADP-glucose formation and the data implied that a concentration of around 0.4mM 3-PGA was necessary to achieve 50 per cent maximal activation ( $A_{0.5}$ ). This  $A_{0.5}$  value was in agreement with results for other leaf ADP-glucose pyrophosphorylases (Sanwal *et al.*, 1968) but was 4 times higher than that previously estimated for the wheat leaf enzyme (McDonald and Strobel, 1970). However these workers obtained their data by assaying the enzyme in the reverse direction i.e. formation of G1P.

The relatively high levels of phosphoglycerate kinase (3-PGA

kinase) present in the leaf preparation may have affected the 3-PGA activation curve, since there was considerable potential for the ATP-dependent conversion of 3-PGA to glycerate-1, 3-bis phosphate (section 5.2.4.4). However subsequent investigation of ATP-dependent ADP-glucose pyrophosphorylase initial reaction velocities in the endosperm and leaf preparations gave similar calculated  $K_m$  (ATP) values. If 3-PGA kinase had been significantly active during the assay of leaf ADP-glucose pyrophosphorylase then it may have been reasonable to expect a relatively high  $K_m$ (ATP) value, since the loss of substrate (ATP) at low concentrations would have the same effect as competitive inhibition i.e. an increase in apparent  $K_m$ . However the situation would be complicated by a loss of activator (3-PGA) at the same rate.

Both endosperm and leaf ADP-glucose pyrophosphorylases exhibited hyperbolic curves of initial reaction velocity as a function of varying ATP concentrations, with the latter enzyme having a requirement for 3-PGA similar to that observed for the leaf GlP-dependent activity. This requirement for 3-PGA was almost absolute, since when the activator was omitted, the leaf enzyme was effectively inactive in respect of its response to varying ATP concentrations in the assay digest. This was not quite the case when GlP was varied, since in the absence of 3-PGA there was some activity.

Levels of ATP in developing endosperms were only determined at 2 ages and approximated to a concentration of 0.3mM at the commencement of dry weight accumulation. Clearly this single determination is insufficient for a reasonable comparison with the observed  $K_m$  (ATP) values for endosperm ADP-glucose pyrophosphorylase.

Nucleoside diphosphate kinase activity was present in both the endosperm and leaf ammonium sulphate preparations and this activity was most likely to be responsible for the apparent 'activation' of ADP-glucose pyrophosphorylase in both preparations when UDP (1mM)

was added to respective enzyme digests. The activity of nucleoside diphosphate kinase would be expected to operate in the direction of UDP synthesis when UTP and ADP were present. These conditions are compatible with the regeneration of UDP for the sucrose synthase reaction and are consistent with the possible role of UDP-glucose pyrophosphorylase in the breakdown of UDP-glucose (Turner, 1969; Turner and Turner, 1975).

Inorganic pyrophosphatase was also present in both preparations and may be important for the hydrolysis of PPi produced in the ADP-glucose pyrophosphorylase reaction. During the course of this work Kumar and Singh (1983) assayed inorganic pyrophosphatase and ADP-glucose pyrophosphorylase in developing wheat grains. While their data was derived from whole grain homogenates, it is interesting to note that the developmental patterns of both enzymes were similar. If the activity of endosperm inorganic pyrophosphatase was assigned to the amyloplast rather than the cytosol, then this would be indicative of a role in maintaining the equilibrium of the ADP-glucose pyrophosphorylase reaction in the direction of ADP-glucose synthesis. Recent evidence suggests that the latter reaction is mainly confined to the amyloplast (McDonald and ap Rees, 1983; Escheverria *et al.*, 1985). This evidence has been obtained from 'amyloplast' preparations derived from soybean and maize endosperm protoplasts. While there is still no adequate visual evidence of reasonable numbers of intact amyloplasts, these experiments, particularly those carried out by Escheverria *et al.* (1985), do indicate that various enzymes, including ADP-glucose pyrophosphorylase and branching enzyme are associated with amyloplasts. However it is not clear whether these enzymes are membrane-bound or lie within the amyloplast as freely soluble enzymes. In the present work, ADP-glucose pyrophosphorylase was extracted, relatively easily, at high stable activity in the crude homogenate. This suggests that the enzyme was freely soluble, since particulate or membrane bound enzymes generally require stronger extraction techniques (Scopes, 1984b). Re-extraction of the pellet (section 5.2.3) with homogenisation buffer containing 0.1 per cent

v/v Triton X 100 followed by assay (section 5.2.4.1) of a supernatant fraction prepared by the procedures outlined in section 5.2.3, did not result in any additional endosperm ADP-glucose pyrophosphorylase activity.

As has been stated previously, both ADP-glucose and PPi inhibited the activity of endosperm ADP-glucose pyrophosphorylase and this was in agreement with the observations of other workers (Preiss *et al.*, 1985). In the present work, the concentrations of ADP-glucose and PPi required to inhibit the ATP-dependent initial reaction velocity by 50 per cent ( $I_{0.5}$ ) were each approximately 0.75mM. However PPi was a more efficient inhibitor than ADP-glucose, achieving 100 per cent inhibition at 4mM, while ADP-glucose achieved only 80 per cent inhibition at 4mM.

The levels of ADP-glucose found in wheat endosperms (section 3.3.1) were fairly constant throughout the period of rapid dry weight accumulation with an estimated endosperm concentration of 0.25mM. This was about one-third of the determined  $I_{0.5}$  (ADP-glucose) value and suggests that ADP-glucose might not be important with respect to *in vivo* regulation of endosperm ADP-glucose pyrophosphorylase activity. However the true *in vivo* concentration of ADP-glucose may be significantly higher than the whole endosperm estimates given here.

The effect of Pi on ATP-dependent endosperm ADP-glucose activity was particularly interesting since the evidence obtained during the broad screen of metabolites implicated a lack of inhibition by this metabolite. In contrast the leaf enzyme was readily inhibited by Pi. When the initial experiment was extended to examine the effect of varying concentrations of Pi on endosperm ADP-glucose pyrophosphorylase activity, an apparent lack of inhibition was again observed. This lack of inhibition, particularly at Pi concentrations greater than 1mM, may have been an artefact resulting from inefficient hydrolysis of  $[U-^{14}C]$  GlP during the alkaline phosphatase digest. However since PPi is also a substrate



for alkaline phosphatase (Bergmeyer, 1974a) it is argued that during the experiments with PPI (when inorganic pyrophosphatase was excluded from the assay digest) there may have been up to 8mM Pi present at some point during the alkaline phosphatase digest. Since 4mM PPI completely inhibited endosperm ADP-glucose pyrophosphorylase activity this implies that alkaline phosphatase was able to hydrolyse all the unreacted  $[U-^{14}C]$  GlP in the presence of 4 mM PPI, since only insignificant counts were retained by the DEAE-cellulose paper strip.

The lack of inhibition of endosperm ADP-glucose pyrophosphorylase by Pi together with an apparent slight rise in activity between 2 and 4mM Pi seems to suggest that Pi may in fact have a stabilising effect on this enzyme. Clearly this suggestion was contrary to previous work on plant and bacterial ADP-glucose pyrophosphorylases (Preiss, 1973; Preiss, 1978; Preiss 1982b) although most of that evidence derives from tissues associated with photosynthesis. Wheat leaf ADP-glucose pyrophosphorylase (McDonald and Stobel, 1970) was inhibited by Pi and Tovey and Roberts (1970) have claimed a similar result for the same enzyme activity as extracted from whole wheat flour. Since such an extract derives from a mixture of chlorophyllous and non-chlorophyllous tissues (pericarp, testa, embryo and endosperm) with different genetic origins, inhibition by Pi cannot be specifically assigned to endosperm ADP-glucose pyrophosphorylase. Additionally Tovey and Roberts (1970) required Pi at 20mM to achieve a 67 per cent inhibition of the control activity. At 2mM Pi, the inhibition was minimal, with the enzyme exhibiting 96 per cent of the control activity.

The levels of Pi in wheat endosperms were not determined during the course of this work, however Duffus and Rosie (1975) have measured the amounts of phosphorus in developing barley endosperms. These amounts were approximately 100  $\mu$ g per endosperm during the period of rapid dry weight accumulation and relate to an endosperm phosphorus concentration of about 160mM. Sofield *et al.* (1977b) have estimated the levels of phosphorus in developing wheat grains

but their data was obtained from acid digests of whole wheat grains and cannot be representative of endosperm phosphorus content. Neither Duffus and Rosie (1975) nor Sofield et al. (1977b) were able to determine how phosphorus may have been partitioned between inorganic and organic phosphate in developing barley endosperms and wheat grains respectively, although both groups showed rising phosphorus levels during dry weight accumulation. Clearly in view of the claims of some workers that Pi inhibits all ADP-glucose pyrophosphorylases (Preiss et al., 1985) it would be interesting to investigate the levels of Pi in tissues (other than those where photosynthesis occurs) where Pi inhibition of ADP-glucose pyrophosphorylase has been observed.

In view of the problems encountered with instability of dialysed endosperm ADP-glucose pyrophosphorylase, there was a possibility that the missing component may have been Pi. The effect of increasing the concentrations of  $MgCl_2$ , DTE and BSA in the enzyme digests showed that the enzyme was dependent upon  $MgCl_2$  for activity. However increasing the concentration of  $MgCl_2$  greater than 5mM or adding varying amounts of DTE or BSA did not enhance or stabilise the activity of the enzyme. In the absence of  $MgCl_2$  the enzyme was essentially inactive and a  $MgCl_2$  concentration of approximately 1mM was necessary for half maximal activity. This result was in agreement with the original work on wheat flour (Espada, 1962). The observed apparent inhibition by  $MgCl_2$  at concentrations greater than 5mM was most likely due to the salt-loading effect previously mentioned in relation to the need for dialysis prior to assaying the enzyme (section 5.4.1).

When aliquots of the dialysed endosperm ADP-glucose pyrophosphorylase were incubated with various salt combinations the highest retention of activity was achieved when Pi was present. Although the concentration of this salt (and the others) was 20mM in the dialysate the subsequent concentration in the assay digest was 2mM. While it can be argued that the effect of Pi was merely to inhibit the activity of alkaline phosphatase, thus increasing the



retained counts, the previously discussed experiments with PPI are in favour of the phenomenon being that of a true stabilisation.

The inclusion of Pi, arsenate, sulphate and molybdate ions in individual dialysate buffers confirmed the above stabilisation. Since these ions were present in their respective buffers at 10mM, their subsequent concentrations in the assay digests were 0.5mM (5  $\mu$ l aliquots were assayed in a total digest volume of 100  $\mu$ l). Again the highest activity was observed with Pi. Complete inhibition was observed with the molybdate dialysate and this was not surprising in view of the ability of molybdate to complex phosphate (Nelson, 1944).

The results from these later experiments, in conjunction with the earlier observations, suggest that Pi is necessary for the activity of endosperm ADP-glucose pyrophosphorylase. This activity was quite stable in the crude extracts and it is hypothesised that the loss of activity on dialysis was due to removal of phosphate. This phosphate may be organic phosphate, however the data in this work seem to implicate inorganic phosphate. Recent reports (I.C. Bridges, personal communication) indicate that stable purified wheat endosperm ADP-glucose pyrophosphorylase has been obtained by subjecting the dialysed ammonium sulphate extract to fast protein liquid chromatography (FPLC) and collecting the eluate in phosphate buffer.

In the present work, the contrasting effects of PPI and Pi on the in vitro activity of endosperm ADP-glucose pyrophosphorylase suggests that the metabolism or turnover of these compounds might be implicated in the regulation of endosperm starch biosynthesis in wheat. The relatively rapid inhibition of endosperm ADP-glucose pyrophosphorylase by PPI implies that a build-up of this ATP-dependent pyrophosphorylase reaction product might feedback - inhibit the activity of the enzyme, regardless of how rapidly ADP-glucose was metabolised by starch synthase. On the other hand, hydrolysis of PPI to 2Pi by inorganic pyrophosphatase would allow

the ATP-dependent pyrophosphorylase reaction to proceed in the direction of ADP-glucose synthesis. The levels of inorganic pyrophosphatase present in the crude endosperm homogenate were estimated to be 3 times greater than ADP-glucose pyrophosphorylase activity, although this estimate was only made at 25 days after anthesis i.e. the age of endosperms taken for large scale preparations (section 5.2.3). Additionally there is no evidence as to how the two activities may or may not be partitioned in the endosperm cell. Association of the activities might facilitate hydrolysis of PPi, while separate compartmentalisation would hinder hydrolysis unless PPi was translocated across, for example, a membrane barrier. If the compartments were the amyloplast and cytosol respectively, then the mechanism of translocation might be important in determining the activity of endosperm ADP-glucose pyrophosphorylase. Clearly removal of PPi by translocation might alleviate the requirement for inorganic pyrophosphatase, since it is conceivable that PPi could be utilised by UDP-glucose pyrophosphorylase (Turner and Turner, 1975). However in the current work, inorganic pyrophosphatase was readily detectable, and therefore should act to hydrolyse PPi, although this could be PPi deriving from other pyrophosphorylase reactions (Feingold, 1982).

UDP-glucose pyrophosphorylase has been implicated in the catabolism of UDP-glucose rather than its synthesis (section 3.4.2) and this reaction would be PPi-dependent. Compartmentation of ADP-glucose pyrophosphorylase and inorganic pyrophosphatase in the amyloplast might therefore mean a role for an enzyme in the regeneration of PPi, presumably in the cytosol. Such an enzyme, pyrophosphate-F6P 1-phosphotransferase (EC 2.7.1.90) and abbreviated to PFK (PPi), catalyses the formation of PPi and F6P from Pi and F-1, 6-bisP and has been detected in developing pea embryos (Edwards and ap Rees, 1986). Clearly developing pea embryos are quite different from developing wheat endosperms and it is not possible to make a direct comparison. Presently, PFK (PPi) has not been assayed in wheat endosperms and this may be an area for future research.

## 5.5 Conclusions

ADP-glucose pyrophosphorylase activity in developing wheat endosperms was apparently of a similar pattern and magnitude to that previously observed for sucrose synthase (section 4.3.1). However in the case of the latter enzyme, the determined levels of substrate(s) (sucrose and UDP) present in the endosperm (section 3.3.1) together with the kinetic data (section 4.3.3) imply that sucrose synthase activity in vivo may be rate-limiting with respect to the catabolism of sucrose for starch biosynthesis. This was not the situation with endosperm ADP-glucose pyrophosphorylase, where substrate levels, at least G1P (section 3.3.1), were complementary to the observed requirements for efficient rates of initial reaction velocity. Since the studies of endosperm ADP-glucose pyrophosphorylase initial reaction velocities were carried out on dialysed ammonium sulphate preparations, it may be incorrect to extrapolate these studies to crude homogenate activities, let alone in vivo rates of catalysis. However the very nature of the dialysed ammonium sulphate preparations, i.e. in relation to their instability, suggests that the calculated  $K_m$  values might be overestimated, although the loss of activity as a function of time after dialysis would not necessarily mean higher  $K_m$  values. Clearly, initial reaction velocities depend upon the concentration of active catalytic sites and the calculated maximum velocity cannot be a constant specific to a particular enzyme, as is the case with  $K_m$  values (M.V. Park, personal communication).

The axiom that starch biosynthesis in all plant cells is regulated at the level of ATP-glucose synthesis by metabolites such as 3-PGA, Pi and PPi (Preiss et al., 1985) may not hold completely true, at least for ADP-glucose pyrophosphorylase extracted from wheat endosperms. The evidence obtained here shows adequate levels of activity in the absence of any added 3-PGA and this was in direct contrast to the analogous activity studied in wheat leaf preparations, which was essentially dependent upon the presence of 3-PGA. Moreover the apparent stability of endosperm ADP-glucose

pyrophosphorylase in the presence of  $P_i$  suggests that this enzyme's activity may be controlled by factors other than those presently understood for leaf and bacterial ADP-glucose pyrophosphorylases.

The minimum levels of UDP-glucose pyrophosphorylase activity in developing wheat endosperms exceeded the respective maximum levels observed for either sucrose synthase or ADP-glucose pyrophosphorylase. Since these minimum levels were observed in relatively young endosperms (13 days after anthesis) and only showed a slow increase during dry weight accumulation, then the control of the synthesis of UDP-glucose pyrophosphorylase may not be a critical factor in the regulation of endosperm starch biosynthesis. However the direction in which this enzyme operates, in vivo, might have a bearing on the rate of starch biosynthesis. Clearly, in spite of relatively high levels of UDP-glucose present in wheat endosperms (section 3.3.1), the production of GlP from UDP-glucose (synthesised by sucrose synthase) would be dependent upon the availability of  $PP_i$ . Since  $PP_i$  was found to inhibit ADP-glucose pyrophosphorylase then, if ADP- and UDP-glucose pyrophosphorylases are separately compartmentalised in endosperm cells, the continued synthesis of ADP-glucose requires effective removal of  $PP_i$  from the site of ADP-glucose synthesis. This removal may mean translocation or hydrolysis and translocation. The latter process implicates inorganic pyrophosphatase, phosphate translocation and subsequent re-synthesis of  $PP_i$ . Either or all of these processes may regulate the rate of starch biosynthesis in wheat endosperms.

## CHAPTER SIX

6.0 General conclusions and suggestions for future work

Although the morphological development of wheat endosperms is a complex series of physiological events, it was possible to design a relatively simple sampling system which ensured that comparable tissues were studied throughout the length of this current investigation. During the course of this study it became quite clear that many previous workers were either unaware or dismissive of the genetic origins of the various tissues that make up the wheat caryopsis. Clearly, such a situation invalidates much of that previous work and this is unfortunate, particularly since the general morphology of wheat caryopsis development has been reasonably well understood for at least the past 80 years. It must be concluded that a biochemical study which purports metabolic function should clearly define the tissue(s) of origin.

The estimations of metabolites and enzyme activities were based on whole tissue homogenates and although the starting tissues were of similar origin, the determined results cannot possibly take into account any localised concentration effects. To this end the isolation of the major organelles implicated in endosperm starch biosynthesis, i.e. the amyloplasts, should be a major research priority for the future.

Although the levels of sucrose per endosperm varied only slightly during development, the estimations of endosperm sucrose concentrations together with the observed initial reaction velocities of sucrose synthase implied that sucrose levels in vivo might rate-limit the conversion of sucrose to UDP-glucose and fructose. Preliminary evidence of a fine control of sucrose synthase activity was observed when UDP was present at less than 0.4mM and, since UDP concentrations of less than 0.4mM were compatible with the estimated levels of endosperm UDP, it is

concluded that the availability of UDP might exert a fine control on the activity of sucrose synthase. The enzyme systems that may be involved in the generation of endosperm UDP were not thoroughly investigated during the course of the present work and this would be an interesting area for future study. The apparent allosteric behaviour of endosperm sucrose synthase requires additional investigation, although the method of assay would have to be more sensitive than the reducing sugar method utilised in the present study. A modification of the radio chemical assay, as used for the determination of ADP- and UDP-glucose pyrophosphorylases, should enable sensitive assay of both ADP- and UDP- dependent sucrose synthase activities.

Although the levels of endosperm invertase were not insignificant, the relative amounts of glucose and fructose determined throughout endosperm development implied that hydrolysis of sucrose was not the major pathway of sucrose catabolism. However invertase activity might be important during the early stages of endosperm development and it would be useful to assay this enzyme in very young endosperms i.e. 1-10 days after anthesis. Since these endosperms are extremely fragile it would not be possible to dissect them free from pericarp testa tissue. An alternative procedure may be to use a microsyringe to remove the endosperm contents directly from the caryopsis.

Endosperm ADP-glucose pyrophosphorylase followed a similar activity profile to sucrose synthase and it is concluded that the onset of rapid dry weight accumulation may be a corollary of the synthesis of these enzymes. UDP-glucose pyrophosphorylase activity in wheat endosperms was present at relatively high levels throughout development and this suggests that in this case, enzyme synthesis might not be a major regulatory factor in wheat endosperm starch biosynthesis.

In terms of maximum initial reaction velocities, the in vivo activity of endosperm ADP-glucose pyrophosphorylase was fairly

closely matched to the measured substrate levels in the endosperm. This was in contrast to sucrose synthase activity, where the apparent maximum initial reaction velocities achieved in vitro were considerably higher than those that could be predicted from the levels of relevant endosperm metabolites. During the course of this study it was not possible to determine frequently the levels of ATP in developing endosperms and it was not clear whether the levels of this metabolite would affect the in vivo activity of ADP-glucose pyrophosphorylase. Levels of ATP in developing wheat endosperms have still not been measured and until they are it will not be possible to assess the role of this metabolite in the regulation of endosperm ADP-glucose pyrophosphorylase activity.

There were significant differences between wheat leaf and wheat endosperm ADP-glucose pyrophosphorylases respectively. In particular the activity of the former enzyme was stable in both crude homogenates and following partial purification by ammonium sulphate fractionation but was dependent upon the presence of 3-PGA for reasonable levels of activity. On the other hand the latter enzyme was stable in crude homogenates but rapidly lost activity following dialysis of the partially purified ammonium sulphate fraction. The endosperm enzyme appeared to require the presence of  $P_i$  for stability (and  $MgCl_2$  for activity) and it was concluded that removal of this metabolite during dialysis was the reason for the loss in enzyme activity. The differences between the two enzymes suggest that they are tissue-specific and may have different structural conformations. This conclusion has, to a certain extent, been confirmed by some recent work (Krishnan et al., 1986) who have shown that in vitro translation products of wheat leaf and endosperm mRNAs respectively gave polypeptides of differing size. However these polypeptides were visualised as a result of denaturing conditions (SDS-PAGE) and there was no evidence that these in vitro translation products were active with respect to the synthesis of ADP-glucose.

Clearly, the realisation that  $P_i$  might act to stabilise the



activity of endosperm ADP-glucose pyrophosphorylase requires further investigation since it was not possible to repeat some of the previously unsuccessful chromatography procedures. This is an obvious area for future work, particularly since successful purification of wheat endosperm ADP-glucose pyrophosphorylase might enable the production of tissue-specific antisera. Presently spinach leaf ADP-glucose pyrophosphorylase antisera has to be used to identify wheat mRNA translation products (Krishnan et al., 1986).

The way in which PPi and Pi are metabolised may lead to a better understanding of how starch biosynthesis in wheat endosperms is regulated. Since PPi was observed to inhibit efficiently the activity of endosperm ADP-glucose pyrophosphorylase it was concluded that hydrolysis or translocation of this reaction product would be essential for continued rates of ADP-glucose synthesis. The high activity of UDP-glucose pyrophosphorylase might catalyse the PPi-dependent cleavage of UDP-glucose to GlP and UTP. If this were the case then the regulation of starch biosynthesis might be PPi-dependent. If PPi were not directly translocated from the amyloplast but first of all hydrolysed by inorganic pyrophosphatase, then it is concluded that a mechanism for the re-synthesis of PPi would be essential. This re-synthesis may involve the enzyme PFK(PPi), and since this enzyme has presently not been assayed in wheat endosperms this is an important area for future work.

Finally, starch biosynthesis is not a particularly popular area for current research. This is surprising in view of the current obsession with biotechnology. The starch industry epitomizes the use of biological processes to produce a vast range of processed, fermented and distilled products and this is in addition to the use of starch for direct nutritional purposes. Popular arguments that current grain surpluses preclude any further research relating to improving yields of cereals are substantially invalid, since any work relating to improved efficiency of starch production should be



continued, even if this eventually means that either less fertilisers are required or that equivalent grain tonnages can be grown on smaller areas of land.

## BIBLIOGRAPHY

Ames, B. N. (1966) In Methods in Enzymology (Neufeld, E.F. and Ginsburg, V., eds.) vol. VIII, pp. 115-116, Academic Press, New York

Amir, J. and Cherry, J.H. (1972) *Plant Physiol.* 49, 893-897

Avigad, G. (1982) In Encyclopedia of Plant Physiology (Pirson, A and Zimmerman, M. H., eds.) New Series, vol. 13A, pp. 217-347, Springer-Verlag, Berlin

Avigad, G. and Milner, Y. (1966) In Methods in Enzymology (Neufeld, E. F and Ginsburg, V., eds.) vol. VIII pp. 341-345, Academic Press, New York

Banks, W. and Greenwood, C. T. (1975) In Starch and its Components pp. 242-306, Edinburgh University Press, Edinburgh

Baxter, E. D. (1972) Ph.D. Thesis, University of Edinburgh

Baxter, E. D. and Duffus, C. M. (1973) *Phytochem.*, 12, 1923-1928

Bergmeyer, H. U. (1974a) In Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd edition, vol. 1, pp. 496-497, Verlag Chemie Weinheim, Academic Press, New York

Bergmeyer, H. U. (1974b) In Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd edition, vol. 1, pp. 505-507, Verlag Chemie Weinheim, Academic Press, New York

Bergmeyer, H. U. (1974c) In Methods in Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd edition, vol. 1, pp. 502-503, Verlag Chemie Weinheim, Academic Press, New York

Bergmeyer, H. U. (1974d). In Methods in Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd edition, vol. 1, p. 508, Verlag Chemie Weinheim, Academic Press, New York

Boehringer Mannheim GmbH (1983) In Methods of Enzymic Food Analysis pp. 47-48, Boehringer Mannheim GmbH, Biochemica, Mannheim, West Germany

Briarty, L. G., Hughes, C. E. and Evers, A. D. (1979) *Ann. Bot.*, 44, 641-658

Buttrose, M. S. (1960) *J. Ultrastructure Research*, 4, 231-257

Buttrose, M. S. (1963) *Aust. J. Biol. Sci.*, 16, 305-317

Caley, C. Y. (1986) Ph.D. Thesis, University of Edinburgh

Chevalier, P. and Lingle, S. E. (1983) *Crop Science*, 23, 272-277

Cochrane, P. (1983) *Aust. J. Plant Physiol.*, 10, 473-491

Cochrane, P. (1985) *Journal of Experimental Botany*, 36, 770-782

Cochrane, M. P. and Duffus, C. M. (1979) *Ann. Bot.*, 44, 66-72

Cook, H. and Oparka, K. J. (1983) *Plant, Cell and Environment* 6, 239-242

Crabtree, B., Leech, A. R. and Newsholme, E. A. (1978) In Techniques in Metabolic Research B211, pp. 1-37, Elsevier/North Holland Scientific Publishers Ltd., Amsterdam

Dale, E. M. and Housley, T. L. (1986) *Plant Physiol.*, 82, 7-10

Dennis, D. T. and Miernyk, J. A. (1982) *Ann. Rev. Plant Physiol.*, 33, 27-50

Dickinson, D. B. and Preiss, J. (1969a) *Arch. Biochem. Biophys.*, 130, 119-128

Dickinson, D. B. and Preiss, J. (1969b) *Plant Physiol.*, 44, 1058-1062

Dixon, M. and Webb, E. C. (1979) In Enzymes 3rd ed., pp. 47-206, Longman Group Ltd., London

Donovan, G. R., Lee, J. W and Hill, R. D (1976) *Cereal Chem.*, 54, 638-645

Duffus, C. M. (1979) In Recent Advances in the Biochemistry of Cereals (Laidman, D. L and Wyn Jones, R. G., eds.) pp. 209-238, Academic Press, London

Duffus, C. M. (1984) In Storage Carbohydrates in Vascular Plants (Lewis, D. H., ed.) pp. 231-252, Cambridge University Press, Cambridge, England

Duffus, C. M. and Cochrane, M. P. (1982) In Physiology and Biochemistry of Seed Development, Dormancy and Germination (Khan, A. A., ed.) pp. 43-66, Elsevier, Amsterdam

Duffus, C. M. and Rosie, R. (1976) *J. Agric. Sci., Camb.*, 86, 627-632

Escheverria, E., Boyer, C., Lie, K-C and Shannon, J. (1985) *Plant Physiol.*, 77, 513-519

Edwards, J. and ap Rees, T. (1986) *Phytochemistry*, 25, 2033-2039

Espada, J. (1962) *J. Biol. Chem.*, 237, 3577-3581

Espada, J. (1966) In Methods in Enzymology (Neufeld, E. F. and Ginsburg, V., eds.) vol. VIII, pp. 259-262, Academic Press, New York

Evers, A. D. (1970) *Ann. Bot.*, 34, 547-555

FAO Production Yearbook (1984) vol. 38, Food and Agriculture Organisation of the United Nations, Rome

Feingold, D. S. (1982) In Encyclopedia of Plant Physiology (Pirson, A. and Zimmerman, M. H., eds.) New Series vol. 13A, pp. 3-58, Springer Verlag, Berlin

Ferdinand, W. (1976) In The Enzyme Molecule pp. 32-35, John Wiley and Sons Ltd., England

Fuchs, R. L. and Smith, J. D. (1979) *Biochim. Biophys. Acta.*, 566, 40-48

Ghosh, H. P. and Preiss, J. (1966) *J. Biol. Chem.*, 241, 4491-4504

Hanes, C. S. (1932) *Biochem J.*, 26, 1406-1421

Heber, U. and Heldt, H. W. (1981) *Ann. Rev. Plant. Physiol.*, 32, 139-168

Henderson, P. J. F. (1978) In Techniques in Protein and Enzyme Biochemistry. B 113, pp. 1-43, Elsevier/North-Holland Scientific Publishers Ltd., Amsterdam

Herold, A. (1980) *New Phytol.*, 86, 131-144

Hofstee, B. H. J. (1952) *J. Biol. Chem.*, 199, 357-364

Hopper, J. E. and Dickinson, D. B. (1972) *Arch. Biochem. Biophys.*, 148, 523-535

- Hughes, C. E. and Briarty, L. G. (1976) *Die Starke*, 28, 336-337
- James, D. W, Preiss, J. and Elbein, A. D. (1985) In The Polysaccharides (Aspinall, G. O., ed.) vol. 3, pp. 107-207, Academic Press, New York
- Jenner, C. F. (1968) *Plant Physiol.*, 43, 41-49
- Jenner, C. F. (1974) *Royal Society of New Zealand Bulletin*, 12, 901-908
- Jenner, C. F. (1982) *Aust. J. Plant Physiol.*, 9, 69-82
- Jenner, C. F. (1986) In Phloem Transport (Cronshaw, J., Lucas, W. J and Giaquinta, R. T., eds.) pp. 561-572, Alan R. Liss Inc., New York
- Jenner, C. F. and Rathjen, A. J. (1972) *Ann. Bot.*, 36, 743-754
- Jenner, C. F. and Rathjen, A. J. (1975) *Aust. J. Plant Physiol.*, 2, 311-22
- Jenner, C. F. and Rathjen, A. J. (1977) *Aust. J. Plant Physiol.*, 4, 691-701
- Kent, N. L. (1983a) In Technology of Cereals 3rd edition, pp. 17-26, Pergamon Press, Oxford
- Kent, N. L. (1983b) In The Technology of Cereals 3rd ed., pp. 27-47, Pergamon Press, Oxford
- Krishnan, H. B., Reeves, C. D. and Okita, T. W. (1986) *Plant Physiol.*, 81, 642-645
- Kumar, R. and Singh, R. (1980) *Phytochemistry*, 19, 2299-2303

Kumar, R. and Singh R. (1981) J. Sci. Food Agric., 32, 229-234

Kumar, R. and Singh, R. (1983) Phytochemistry, 22, 2405-2407

Kumar, R. and Singh, R. (1984) J. Agr. Food Chem., 32, 806-808

Langer, R. H. M. (1979) In How Grasses Grow pp. 1-3, Edward Arnold, London

Lee, D. R. and Atkey, P. T. (1984) Can. J. Bot., 62, 1319-1326

Lee, P. D. and Su, J. C. (1982) Proc. Nat. Sc. Council (Taiwan), 6, 189-196

Lineback, D. R. (1984) In Cereal Polysaccharides in Technology and Nutrition (Rasper, V. F., ed.) pp. 1-21, American Association of Cereal Chemists, St. Paul, Minnesota

Lineweaver, H. and Burk, D. (1934) J. Amer. Chem. Soc., 56, 658-666

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem., 193, 265-275

MacDonald, F. D and ap Rees, T. (1983) Biochem. Biophys. Acta, 755, 81-89

MacDonald, P. W. and Stobel, G. A. (1970) Plant Physiol., 46, 126-135

MacMasters, M. M., Hinton, J. J. C. and Bradbury, D. (1971) In Wheat Chemistry and Technology (Pomeranz, Y., ed.) vol III, pp. 51-113, American Association of Cereal Chemists, St. Paul, Minnesota

Manners, D. J. (1974) In Essays in Biochemistry (Campbell, P. N and Dickens, F., eds.) vol. 10, pp. 37-71, Academic Press, London

- Mares, D. J., Norstog, K. and Stone, B. A. (1975) Aust. J. Bot., 23, 311-326
- May, L. H. and Buttrose, M. S. (1959) Aust. J. Biol. Sci., 12, 146-159
- Meredith, P. and Jenkins, L. D. (1976) Starke 19, 115-123
- Morell, M. and Copeland, L. (1985) Plant Physiol., 78, 149-154
- Murata, T. (1972) Agr. Biol. Chem., 36, 1815-1818
- Nasyrov, Y. S. (1978) Ann. Rev. Plant Physiol., 29, 215-237
- Nelson, N. (1944) J. Biol. Chem., 153, 375-380
- Nelson, O. E. (1980) In Advances in Cereal Science and Technology (Pomeranz, Y., ed.) vol. III, pp. 41-71, American Association of Cereal Chemists, Inc., St. Paul, Minnesota
- Nomura, T. and Akazawa, T. (1973) Arch. Biochem. Biophys., 156, 644-652
- Oparka, K. J. and Gates, P. (1981) Planta, 152, 388-396
- Parker, M. L. (1985) Journal of Cereal Science, 3, 271-278
- Passonneau, J. B., Lust, W. D. and McCandless, D. W. (1978) In Techniques in Metabolic Research B 212, pp. 1-27, Elsevier/North Holland Scientific Publishers Ltd., Amsterdam
- Peacock, W. J., Gerlach, W. L. and Dennis, E. S. (1983) In Wheat Science-Today and Tomorrow (Evans, L. T and Peacock, W. J., eds.) pp. 41-60, Cambridge University Press, Cambridge



Peng, C. T. (1977) In Sample preparation in liquid scintillation counting RCC Review 17, pp. 13-25, The Radiochemical Centre Ltd., Amersham, Bucks, England

Perez, C. M., Perdon, A. A., Resurreccion, P., Villareal, R. M. and Juliano, B. O. (1975) *Plant Physiol.*, 56, 579-583

Percival, J. (1921) In The Wheat Plant, pp. 100-143, Duckworth and Co., London

Pontis, H. G. and Salerno, G. L. (1980) In Mechanisms of Saccharide Polymerisation and Depolymerisation (Marshall, J. J., ed.) pp. 31-42, Academic Press, New York

Preiss, J. (1973) In The Enzymes (Boyer, P. D., ed.) vol. VIII, Part A, 3rd ed., pp. 73-119, Academic Press, New York

Preiss, J. (1978) In Advances in Enzymology and Related Areas of Molecular Biology (Meister, A., ed.) vol. 46, pp. 317-381, Wiley, New York

Preiss, J. (1982a) *Ann. Rev. Plant. Physiol.*, 33, 431-454

Preiss, J. (1982b) In Encyclopedia of Plant Physiology (Loewus, F. A and Tanner, W., eds.) New Series, vol. 13A, pp. 397-417, Springer-Verlag, Berlin

Preiss, J., Lammel, C and Sabraw, A. (1971) *Plant Physiol.*, 47, 104-108

Preiss, J. and Levi, C. (1979) In Encyclopedia of Plant Physiology (Gibbs, M and Latzko, E., eds.) vol. 6, pp. 282-312, Springer-Verlag, Berlin

- Preiss, J., MacDonald, F. D., Singh, B. K., Robinson, N. and McNamara, K. (1985) In New Approaches to Research on Cereal Carbohydrates (Hill, R. D and Munck, L., eds.) Progress in Biotechnology, vol. 1, pp. 1-7, Elsevier, Amsterdam
- Reeves, C. D., Krishnan, H. B. and Okita, T. W. (1986) Plant Physiol., 82, 34-40
- Roberts, R. M. and Tovey, K. C. (1970) Anal. Biochem., 34, 582-590
- Rogers, S. O. and Quatrano, R. S. (1983) Amer. J. Bot., 70, 308-311
- Sakri, F. A. K. and Shannon, J. C. (1975) Plant Physiol., 55, 881-889
- Sanwal, G. G., Greenberg, E., Hardie, J., Cameron, E. C. and Preiss, J (1968) Plant Physiol., 43, 417-427
- Saunders, C. E. (1928) Sci. Agr., 8, 524-531
- Scopes, R. K. (1984a) In Protein Purification pp. 43-52, Springer-Verlag, New York
- Scopes, R. K. (1984b) In Protein Purification pp. 34-37, Springer-Verlag, New York
- Shannon, J. C. (1978) J. of the Pennsylvania Agr. Exp. Station., 78-94
- Shen, L. and Preiss, J. (1964) Biochem. Biophys. Res. Comm., 17, 424-429
- Sherman, J. R. (1963) Analytical Biochemistry, 5, 548-554

Shewry, P. R. and Mifflin, B. J. (1985) In Advances in Cereal Science and Technology (Pomeranz, Y., ed.) vol VII, pp. 1-5, American Association of Cereal Chemists, St. Paul, Minnesota

Simmonds, D. H. and O'Brien, T. P. (1981) In Advances in Cereal Science and Technology (Pomeranz, Y. ed.) vol. IV pp. 5-70, American Association of Cereal Chemists, Minnesota

Smart, M. G. and O'Brien, T. P. (1983) Protoplasma 114, 1-13

Sofield, I., Evans, L. T., Cook, M. G. and Wardlaw, I. F. (1977a) Aust. J. Plant Physiol., 4, 785-797

Sofield, I., Wardlaw, I. F., Evans, L. T. and Cook, M. G. (1977b) Aust. J. Plant Physiol., 4, 799-810

Sowokinos, J. R. (1981) Plant Physiol., 68, 924-929

Sowokinos, J. R. and Preiss, J. (1982) Plant Physiol., 69, 1459-1466

Su J. C. and Preiss, J. (1978) Plant Physiol., 61, 389-393

Sugimoto, E. and Pizer, L. I. (1968) J. Biol. Chem., 243, 2081

The Biochemical Society (1987) In Policy of the Journal (Biochemical Journal) and Instructions to Authors pp. 12-21, The Biochemical Society, London

Thorne, J. H. (1985) In Annual Review of Plant Physiology (Briggs, W. R., Jones, R. L and Walbot, V., eds.) vol. 36, pp. 317-343, Annual Reviews Inc., USA

Tovey, K. C. and Roberts, R. M. (1970) Plant Physiol., 46, 406-411

Tsai, C. Y. and Nelson, O. E. (1966) Science, 151, 341-343

Tsai, C. Y. Salamini, F. and Nelson, O. E. (1970) *Plant Physiol.*, 46, 299-306

Tsay, J. S., Kuo, W. L. and Kuo, C. G. (1983) *Phytochemistry*, 22, 1573-1576

Turner, J. F. (1969) *Aust. J. Biol. Sci.*, 22, 1321-1327

Turner, J. F. and Turner, D. H. (1975) *Ann. Rev. Plant Physiol.*, 26, 159-186

Turnquist, R. L. and Hansen, R. G. (1973). In The Enzymes (Boyer, P. D., ed.) 3rd edition, vol. VIII, part A, pp. 51-71, Academic Press, New York

Walpole, R. E. (1982) In Introduction to Statistics 3rd edition, pp. 346-384, Macmillan Publishing Company, New York

Watson, P. A. (1985) Ph.D Thesis, University of Edinburgh

Zadoks, J. C., Chang, T. T. and Konzak, C. F. (1974) *Weed Research*, 14, 415-421